HISTOLOGICAL TECHNIQUE

FOR NORMAL TISSUES, MORBID CHANGES AND THE IDENTIFICATION OF PARASITES

H. M. CARLETON

OXFORD MEDICAL PUBLICATIONS

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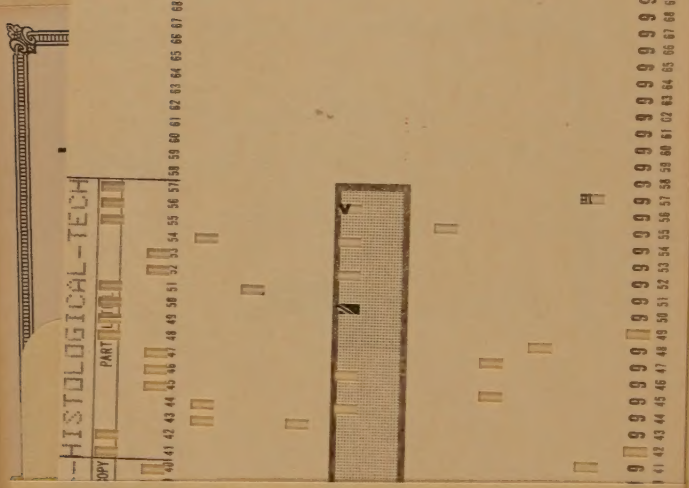

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FOR NORMAL TISSUES, MORBID CHANGES AND THE IDENTIFICATION OF PARASITES

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HUMPHREY MILFORD

OXFORD UNIVERSITY PRESS

London Edinburgh Glasgow Copenhagen
New York Toronto Melbourne Cape Town
Bombay Calcutta Madras Shanghai

1926

QM
555
C3

First Printed 1926

PRINTED IN GREAT BRITAIN BY MORRISON AND GIBB LTD., EDINBURGH

TO

SIR CHARLES SHERRINGTON, O.M., F.R.S., M.D.

WAYNFLETE PROFESSOR OF PHYSIOLOGY
IN THE UNIVERSITY OF OXFORD

Biology

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INTRODUCTION

THE aim of this book is to give, in a compact form, the chief methods employed in the microscopical examination of human and other mammalian organs.

A departure from current practice has been made by inserting in Part I. of this book two chapters on the cell in relation to Histological Technique. These chapters outline the conception of cells and tissues as complex, colloidal entities, which are all too easily altered by the treatment to which they are subjected. The importance of this conception cannot be over-emphasised. The worker in Biology or Medicine must be a Pygmalion, quick to transform the cold marble of his technical result into the glow of the living cell. To further this the question of artefacts in fixed and stained preparations is also stressed.

To include *all* the methods of normal and morbid histology in a manual of moderate size is manifestly impossible. The following plan has therefore been adopted in these pages :

Firstly, to describe in detail (in Part II.) the fundamental processes of Fixation, Embedding, Section Cutting, and Staining. With regard to fixation and staining, a number of "Type Methods" of wide application have been chosen. Once these have been mastered by the beginner, the application of the special methods should be easy.

Secondly, to provide workers with accessory and special methods (Parts III. and IV.). The methods most suitable for the various organs and tissues are dealt with here. They are necessarily a selection, and by no means represent the totality

of the technical approaches known. It has thus been necessary to adapt the length of the sections dealing with the methods for the organs and tissues to the importance of the subject. That is why the technique for the microscopic study of the Spleen is described in a few paragraphs, while that for the Nervous System entails many pages.

Thirdly, to set out (in Part V.) the methods for the identification of morbid changes, or parasites of various sorts, in tissues. Whenever such changes, or organisms, have important clinical bearings, the technique most suitable for their identification is detailed. Methods for examining the body fluids and dejecta for parasites have also been included.

A word regarding the "Notes" which are often appended to the various methods. A successful histological technique depends largely on one's knowledge of various small "tips." The degree of success attendant upon any manipulative branch of Science—whether it be Surgery, Microscopical Technique, or Mechanics—is largely in proportion to the worker's knowledge of small hidden difficulties, and of the means for avoiding them. To make this book as practical as possible such pitfalls have been freely indicated, even at the risk of seeming superfluous.

A very short and general Bibliography has been appended. It refers chiefly to standard works, or important monographs, in which full bibliographies may usually be found. This manual would have been unduly lengthened by a complete list of references. But the skeleton Bibliography included will enable anybody to get access to the literature.

My acknowledgments are many. To thank Professor Sherrington at all adequately would surpass the limits both of my space and of my ability. All that I can do is to dedicate to him this book. Colonel Robert McCarrison, I.M.S., urged me to undertake this book and together we discussed its inception. I can only hope that he will not regret what I might perhaps call his activating influence when he reads it.

Professor J. P. Hill has always been ready to place his knowledge at my disposal, while Dr. A. G. Gibson's advice has been most valuable in relation to the Pathological chapters of this book. To Mr. F. Haynes, who has collaborated in two of the chapters, I wish to express my gratitude for much help in the preparation of this manual. I also am deeply indebted to my wife and to Dr. Boris Shapiro for much help and many informal suggestions.

My thanks are due to Messrs. Atha, Hawksley & Leitz for permission to copy, or make use of, electrotypes of various instruments.

I would thank the many assistants, in different laboratories, who, at one time and another, have given me practical information such as cannot be acquired from books.

And, finally, it is with pleasure that I thank Mr. Humphrey Milford for the care and speed with which he has produced this book.

H. M. C.

Physiology Laboratory, Oxford,
January 17, 1926.

TABLE FOR THE DILUTION OF 96 PER CENT. ALCOHOL

(This is the concentration (96 to 97 per cent.) of the "Rectified Spirit" supplied to most laboratories.)

Grade Required.	Volumes of Rectified Spirit.	Volumes of Water.
90 per cent.	93·5	6·5
80 ,,	83·3	16·7
70 ,,	72·9	27·1
60 ,,	62·5	37·5
50 ,,	52·1	47·9
40 ,,	41·6	58·4
30 ,,	31·2	68·8

TABLE FOR THE DILUTION OF COMMERCIAL (40 PER CENT.) FORMALIN SOLUTION

Grade of Formol Required.	Volumes of 40 per cent. Formalin.	Volumes of Water or Saline Solution.
3 per cent.	7·5	92·5
5 ,,	12·5	87·5
8 ,,	20·0	80·0
10 ,,	25·0	75·0
15 ,,	37·5	62·5
20 ,,	50·0	50·0

TABLE FOR THE PREPARATION OF VARIOUS GRADES OF ALCOHOL FROM 90 PER CENT. DOWNWARDS (AFTER GAY-LUSSAC)

NOTE.—The intersection of the horizontal row (= grade required) and the vertical row (= original grade) indicates the number of volumes of water required for every 100 *volumes of the original grade of alcohol.*

Weaker Grade Required.	Original Grade.								
	90 per cent.	85 per cent.	80 per cent.	75 per cent.	70 per cent.	65 per cent.	60 per cent.	55 per cent.	50 per cent.
85 per cent.	6·56	..	..	..	..	..	..	..	..
80 ,,	13·79	6·83	..	..	..	..	..	..	..
75 ,,	21·89	14·48	7·20	..	..	..	..	..	..
70 ,,	31·05	23·14	15·35	7·64	..	..	..	..	..
65 ,,	41·53	33·03	24·66	16·37	8·15	..	..	..	..
60 ,,	53·65	44·48	35·44	26·47	17·58	8·76	..	..	..
55 ,,	67·87	57·90	48·07	38·32	28·63	19·02	9·47	..	..
50 ,,	84·71	73·90	63·04	52·43	41·73	31·25	20·47	10·35	..
45 ,,	105·34	93·30	81·38	69·54	57·78	46·09	34·46	22·90	11·41
40 ,,	130·80	117·34	104·01	90·76	77·58	64·48	51·43	38·46	25·55
35 ,,	163·28	148·01	132·88	117·82	102·84	87·93	73·08	58·31	43·59
30 ,,	206·22	188·57	171·05	153·61	136·04	118·94	101·71	84·54	67·45

PART I

STRUCTURE AND COMPOSITION OF THE CELL IN RELATION TO HISTOLOGICAL TECHNIQUE

CHAPTER I

THE CELL AS A STRUCTURAL ENTITY

1. Living Matter — 2. The Cell — 3. Nuclear Components — 4. Cytoplasmic Components—5. Theories of the Structure of Protoplasm—6. Tissues.

1. LIVING MATTER

Before considering the methods of Histological Technique, it is essential to know something of the structure and nature of the substance of which tissues and cells are composed. Strictly speaking, the term "Protoplasm" only applies to those components of the organism so long as they are alive. Once the liberation of those forms of energy which we associate with life has come to an end, once cells or tissues have died, or have been killed by artificial means such as histological fixation, we are no longer dealing with protoplasm in the true sense of the word.

The intimate structure of protoplasm is a very complicated subject, and our knowledge regarding it is still fragmentary. For this reason, the following paragraphs on living matter do not aim at giving even the most cursory summary of its structure and properties. They are only intended to supply information essential to the understanding of microscopical technique.

2. THE CELL

Formerly the cell was regarded as being essentially composed of nucleus, cytoplasm, and centrosome. Modern technique, however, has shown that cell structure is far more complicated than was previously supposed.

The following table shows the more important of the cell constituents, which are diagrammatically illustrated in Fig. 1 :

CELL.

- NUCLEUS
  1. Nuclear membrane.
  2. Chromatin.
  3. Nucleolus
     - Karyosome.
     - Plasmosome.
  4. Karyolymph or nuclear sap.
- CYTOPLASM
  1. Centrosome.
  2. Golgi bodies.
  3. Mitochondria.
  4. Metaplasmic inclusions—*e.g.* fat, glycogen, yolk, etc.
  5. The ground cytoplasm.

3. THE NUCLEAR COMPONENTS

(i) **The Nuclear Membrane.**—In the resting cell the nucleus is limited by a definite membrane, the appearance of which is very variable in different cells. Usually it is thin and appears to be microscopically homogeneous. There seems to be little doubt that it is a definite structural membrane, and not merely an optical effect due to differences in surface tension at the interface between nucleus and cytoplasm. Its shape appears to be due partly to surface tension and osmosis, partly to simple mechanical factors (pressure, traction, etc.), sometimes to the presence of an intranuclear rodlet. The nuclear membrane disappears during mitosis.

(ii) **The Chromatin** of the non-dividing cell forms—according to the classical conception—a definite network. While intravitam observations have shown that this may be true in some cases, much modern research strongly suggests that the chromatin in the living cell is present either in the shape of scattered granules floating freely in the nuclear sap, or else is actually in a state of solution within the latter.[1] During mitosis, however, the chromatin becomes condensed to form the chromosomes, concerning the existence of which there is no doubt.

[1] The brilliant microdissection studies of Chambers seem conclusively to invalidate the idea (derived from the study of fixed cells) of elaborate chromatin and linin meshworks. [See his article in *General Cytology*, referred to in the Bibliography of this book (p. 367).]

(iii) The Nucleolus.—In addition to the chromatin, the nucleus may contain one or more oval or rounded bodies lying

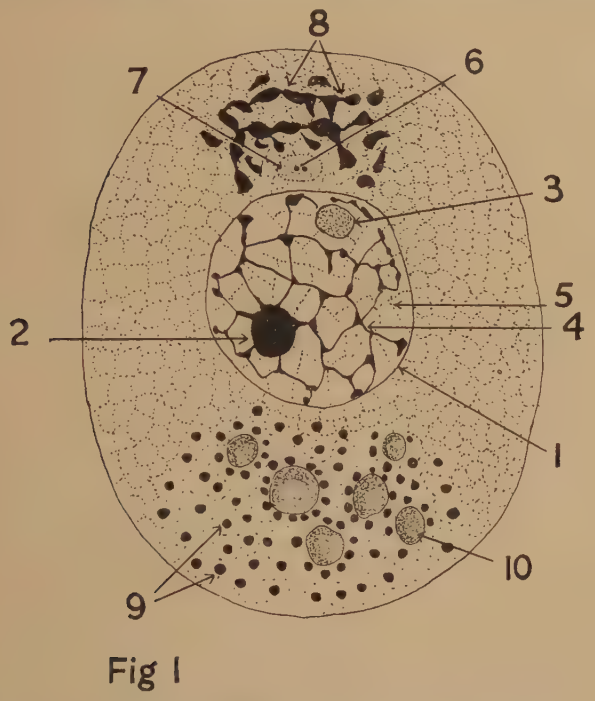


FIG. 1.—SCHEME OF THE CELL AS SEEN AFTER FIXATION.

For the explanation of the following terms, see the text.

1. Nuclear membrane.
2. Karyosome=chromatin nucleolus.
3. Plasmosome=plastin nucleolus (=true nucleolus).
4. Chromatin meshwork.
5. "Linin" meshwork.
6. Centrosomes.
7. Archoplasm.
8. Golgi network (=Golgi apparatus; internal apparatus of Golgi; Golgi bodies, etc.).
9. Mitochondria of the granular type.
10. Metaplasmic inclusions.

The ground cytoplasm, after fixation and staining, often presents the appearance of a fine meshwork, the significance of which is discussed in the text.

freely within the nuclear membrane. These structures, which are the largest of the intranuclear elements, are usually termed

nucleoli. They are peculiar in that they possess the power of independent fission, and are said sometimes to show amœboid movements in the living cell.

Nucleoli may be of two kinds : sometimes they are merely bulky condensations of chromatin, in which case they are termed *karyosomes*. But often they are composed of a substance known as plastin, which differs radically in its chemical and staining reactions from chromatin. Nucleoli of this type (sometimes called " true nucleoli ") are usually known as *plasmosomes*. Finally, some nucleoli contain both chromatin and plastin in varying proportions, while one or more granules—apparently of a special nature and function—have been noted in certain nucleoli. These intranuclear bodies have been called *nucleolini*.

(iv) **The Karyolymph** (sometimes called nuclear sap) is the fluid contained within the nuclear membrane. The chromatin and other intranuclear elements lie in this fluid, which, in the living cell, contains proteids in solution. After fixation the latter may become precipitated to form a coagulum, which can be stained with certain histological dyes.

It is probable that the " *Linin meshwork*," often figured in the diagrams of the cell in nearly all biological text-books, does not exist as such in the vast majority of living cells. It is certain that the structure usually described as the " linin " reticulum is hardly ever revealed by intravitam methods, that its appearance varies enormously according to the fixative employed, and that powerful coagulants of proteids cause a very definite meshwork—differing radically from chromatin in its staining reactions—to appear within the nucleus. On the other hand, methods of fixation which are known to preserve the other cell components in as life-like a condition as possible show no linin reticulum.

It is probable, then, that this structure is, in most, if not in all cases, but an artificial coagulation product derived from the albuminous substances of the karyolymph.

4. THE CYTOPLASMIC COMPONENTS

The cytoplasm is now known to be of great structural complexity. The older histologists described it as a " ground substance " containing little beyond the centrosome and sometimes globules—fatty or otherwise. Their failure to identify the majority of the cytoplasmic inclusions was due partly to their disregard of the intravitam methods of observation, partly to faulty fixation. That is to say, that most of the cytologists of the nineteenth century did not examine cells or tissues until they had been fixed and stained. And their technique was such that the majority of the very delicate cytoplasmic elements were dissolved by the fixative employed. The results obtained by such methods are analogous to the effect of maceration on the carcase of an animal; only the most resistant elements—*i.e.* the skeleton—remain.

(i) **The Centrosome** is present in nearly all the cells of the higher animals, certainly during their embryonic condition. It is represented by a single, or often a pair of small granules. In elongated cells the centrosome usually lies between the nucleus and the free border of the cell; in rounded or polyhedral cells it is close to the nucleus. Often the cytoplasm around the centrosome is relatively dense; in such cases it forms a small oval area known as the *Archoplasm*. Highly specialised cells—and especially those in which the power of reproduction has been lost—are usually devoid of a centrosome in the adult condition. The red blood corpuscle and the nerve cell are familiar examples of this.

(ii) **The Golgi Bodies** are present in most cells, and recent work suggests that they may be included among the most constant components of the cell. In position and shape the Golgi bodies are very variable. Sometimes they occur in fixed and stained specimens as a system of curved rodlets or *dictyosomes* grouped around the archoplasm and the centrosome. The spermatocytes of many animals—*e.g.* the guinea-pig—possess Golgi bodies of this pattern. Sometimes the Golgi elements are disposed so as to form a network, usually localised, in the case of elongated cells, at one pole of the cell. In polyhedral cells the network is usually juxtanuclear, while in the

nerve cell the Golgi bodies occupy the greater part of the cytoplasm, forming an elaborate reticulum completely surrounding the nucleus. During mitosis the Golgi elements break up into rodlets, and these dictyosomes pass into each of the daughter cells. The substance of the Golgi elements is only approximately halved during mitosis ; it does not, in contrast with the chromatin, appear to undergo any accurate quantitative division. Half of the pre-formed rodlets, or dictyosomes, are passively carried into each daughter cell, there being no known instance in which the rodlets themselves divide.

(iii) **The Mitochondria** are nearly invariable constituents of the cell. They appear as round, rod-like, or filamentous bodies in the cytoplasm. Much confusion exists in the nomenclature of these structures. The term *mitochondria* is used by many—and especially the continental authors—to apply to the granular variety only, the elongated forms being called *chondriokonts*, while both types are grouped together under the general denomination of *chondriosomes*. Most British authors, however, use the word mitochondria as a general term, specifying the type (rod-like, granular, etc.), when and where necessary. It is in this sense in which the term mitochondrium is used in this book. During mitosis the mitochondria are approximately divided into two groups, one of which is distributed to each daughter cell. The whole process seems to be rough and ready and purely passive—as with the Golgi bodies. There is, however, one interesting exception : in the male germ cells of the scorpion *Centrurus exilicauda* (as shown by Wilson), the mitochondrial material becomes concentrated into a ring-like formation in the primary spermatocytes. This divides equally during the first and second maturation divisions. The result is that each of the four spermatids receives one-fourth of the original mitochondrial material of the primary spermatocyte. The reason for the equal distribution of the mitochondria in this particular case is quite unknown.

Differentiation between Golgi bodies and mitochondria is based partly on their morphology, partly on their different behaviour towards reagents. The Golgi bodies form either a coarse network or large rods. The mitochondria may be granular, filamentous or rodded, but they are always far smaller than the Golgi bodies.

(iv) **The Metaplasmic Inclusions** are primarily nutritive substances elaborated and stored within the cell. Those which can be optically studied occur as granules or discs of various sizes. *Fat*, *Glycogen*, and *Yolk* are the most important of these reserve products.

Fat occurs in the shape of globules which, in the case of fully developed adipose tissue cells, attain such dimensions as to occupy nearly the entire cytoplasm. Apart from its typical histo-chemical reactions, fat can easily be recognised intra-vitam by its extreme refringency.

In the adult mammal, fat is normally present in the following tissues :

(i) *Adipose Tissue* proper, which is most developed subcutaneously and in the omentum.

(ii) *Liver.*—In this organ the amount of intracellular fat is very variable. In the cat, for instance, there is much, while in the normal adult human being hepatic fat is scanty, except in the newborn child and in lactating women, in which it increases in amount.

(iii) *Interstitial Tissue of the Testis.*

(iv) *Ovarian follicles* in early stages of development.

Glycogen exists in the living cell in a state of solution within minute cytoplasmic droplets of a watery nature. In stained specimens, after fixation in absolute alcohol, it appears as a cloud of fine granules or in amorphous masses.

Practically all tissues contain glycogen at some usually a fœtal— stage of their development. In the adult it is most abundant in the liver and muscles.

Yolk is found in the shape of granules or small discs, and in the living cell is of a yellowish colour. It varies considerably in amount, being present, of course, only in embryonic tissues or ova. Sometimes, as in certain amphibian larvæ, the yolk discs are so large that they deform the nucleus.

(v) **The Ground Cytoplasm and Theories of the Structure of Protoplasm.**—All the cytoplasmic bodies considered hitherto lie in a matrix known as the ground cytoplasm. In living cells this usually appears as a colourless and more or less fluid menstrum in which the mitochondria, Golgi elements, and metaplasmic bodies lie.

Much controversy has raged around the question of the intimate structure of the ground cytoplasm, concerning which four great theories have been evolved, now, however, chiefly of historical interest.

The *Granular Theory* of Altmann held that the ground cytoplasm was composed of innumerable granules lying in a semifluid matrix. These granules, which were too large to be colloidal particles, were probably mitochondrial in nature. Altmann, however, termed these bodies " Bioblasts," and regarded them as the ultimate elements of living matter.

The *Reticular Theory* (Carnoy, etc.) supposed that the protoplasm of the cell was made up of a continuous framework—rather like the fibrous tissue of a sponge—with a fluid in between the meshes. Since the observations on which this theory was based were made largely on fixed material, they are open to criticism. Further, in most fixed and stained cells it is impossible to see such a reticulum.

The *Fibrillar Theory* of Flemming assumed the cell protoplasm to be composed of disconnected threads lying in a fluid (enchylema). These filaments, which Flemming observed in living cells, were possibly mitochondrial in nature.

The *Alveolar Theory* of Bütschli. The ultimate structure of protoplasm is regarded as foam-like, in that it is made up of closely packed droplets of a liquid alveolar substance dispersed in a continuous (also liquid) interalveolar fluid. Bütschli's theory, which still has many followers, was based on studies on living and fixed cells and on artificial emulsions.

Which of these theories is correct ? Bütschli's view is probably the only one which has any counterpart in the living cell, and that only in a limited number of examples. For instance, the eggs of certain invertebrates such as Echinoderms appear to have, both during life and after appropriate fixation, an alveolar structure. But the fact remains that the living cells of vertebrates show no foam-like appearances. When studied with the dark ground illumination their protoplasm is seen to be composed of innumerable colloidal particles. Apart from this it is structureless.

In any case, none of these theories deal with the intimate structure of living matter. In all discussions on this question it should be remembered that protoplasm is a complex associa-

tion of colloids, that it behaves physically and chemically as such, that its activities are largely controlled by its colloidal constitution. Variations in the physiology of the cell are accompanied by changes in the colloidal nature of its protoplasm, while the very change from life to death appears to be characterised by the onset of a gel condition.

(vi) Tissues.—Any collection of cells of more or less the same structure and function is called a tissue. Thus, bone, the various types of epithelia, and muscle each constitute a tissue. It follows from this that it is possible to identify a tissue microscopically by noting the character and arrangement of its constituent cells.

But, apart from this purely morphological method, it is possible—and usually far easier—to distinguish many tissues by their histo-chemical behaviour towards reagents, and especially towards dyes. Thus, collagen and elastic connective tissue can be sharply differentiated from one another by various staining methods. This differential staining is due to the different physico-chemical constitution of the various tissues.

CHAPTER II

THE CELL AS A PHYSICO-CHEMICAL ENTITY

1. Optical Properties of Protoplasm—2. Its Fluidity—3. The Surface Membrane of the Cell—4. Osmosis—5. The Colloidal Conception of Protoplasm—6. Micro-Dissection—7. Autolysis—8. Chemistry of Protoplasm.

1. OPTICAL PROPERTIES OF PROTOPLASM

Optically, protoplasm is a transparent—or more usually translucent—substance. It shows some variation in its degree of refraction, which, though higher than that of water, varies in the different cell constituents. Were this not so, it would be impossible to distinguish these in living cells.

Certain other physical properties of protoplasm must now be noted.

2. FLUIDITY OF PROTOPLASM

With regard to this condition, the fact that the watery or oily droplets (so frequently seen in cells) are always spherical can only be ascribed to surface tension—*i.e.* to the interaction of two immiscible fluids. We have reason to believe, however, that the protoplasm of many cells is in a state intermediate between the fluid and the solid ; it has some of the resistance of the latter combined with several of the properties of the former. This is due to the protoplasm being in what is known as the "gel" condition—a matter to be considered shortly. But the fact remains that the substance of cells is more or less of a fluid nature. Observations of the movements of an amœba or a white blood corpuscle abundantly illustrate this.

3. THE SURFACE MEMBRANE OF THE CELL

All cells possess a surface membrane, which is normally insoluble in water. And this in spite of the fact that living matter is approximately 80 per cent. water by weight. This insolubility is due to the protection afforded by the surface layer, which is generally considered to be of a lipoidal nature. This surface membrane is semipermeable during life ; it permits certain of the substances dissolved in the surrounding medium to pass into the cell, but is a barrier to others. Inversely, the dissolved food products within the cell are retained, although they are soluble in the surrounding medium, whether this be water (as in the case of many protozoa) or a body fluid of the nature of blood or lymph (as in the multicellular organisms). In fact, the processes of absorption, excretion, etc., are primarily dependent on the surface membrane. If this be seriously damaged by physical or chemical means, the cell dies. An amœba, if electrocuted, becomes dissolved in the surrounding water. Many poisons, including some of the bacterial toxins, act directly on the surface membrane of the cells attacked by them.

4. OSMOSIS

If at one side of a membrane we have a solution of a substance at a certain concentration, and at the other side of the membrane a solution of the same substance at a lesser concentration, water will pass through the membrane from the solution of smaller to the solution of greater concentration until the solutions on both sides of the membrane are at the same concentration. This attempt to equalise the concentrations of sols on each side of a membrane is known as *Osmosis*, and it has a very important bearing on the life of the cell. For instance, the cells and tissues of the mammalian body have an osmotic pressure equal to that of 0·9 per cent. sodium chloride solution. Thus, if a mammalian cell—say a red blood corpuscle—be placed in a solution of 0·9 per cent. sodium chloride, the contents of the cell are at the same molecular concentration as the solution surrounding it, and therefore the influx of water into the cell will be exactly balanced by the outgo. Consequently,

the cell will be unchanged in appearance. Suppose now that in place of such an *Isotonic* solution, as it is called, we have a *Hypertonic* solution, *i.e.* one of a higher molecular concentration. In order that the solutions within and without the cell shall be of the same concentration, water must pass from the cell to the surrounding solution, with the result that the cell will shrink. And finally, if we have a *Hypotonic* solution—one of lower molecular concentration than the cells lying in it—water will pass from the solution of low concentration into the cell in an attempt to equalise the osmotic pressures of the solutions inside and outside the cell. The result will be that water will pass into the cell until this is so distended by the water that it bursts.

Osmosis is therefore of great importance to the histologist in the examination of living cells in fluid media if a true picture of the cells be desired. Red blood corpuscles, however, are more sensitive to osmotic variations than other cells.

5. THE COLLOIDAL CONCEPTION OF PROTOPLASM

This is a conception of fundamental importance. A brief review of it is therefore necessary.

In a true solution the dissolved substance is in a state of molecular dispersion in the solvent. In a colloidal solution the particles are not in so fine a state of dispersion, each particle being composed of many molecules. An example will make this clear: Suppose an insoluble solid substance could be reduced by mechanical means to progressively smaller and smaller particles. Before being reduced to its individual atoms it would pass through a state of subdivision in which the particles would be sufficiently small to remain in suspension in a liquid. Now substances in this state of " pseudo-solution " are termed *Colloids*. To the naked eye, and by ordinary methods of microscopic observation, a suspension of this kind appears to be perfectly homogeneous—provided that the particles are sufficiently small. Indian ink and gamboge are examples of this. Both are formed of a solid phase dispersed in a liquid phase, and they belong to the group of colloids called *Suspensoids*. Often, however, a colloid consists of two immiscible

fluids, one of which forms the continuous, and the other the dispersed, phase. Such a colloid is known as an *Emulsoid.* It is necessary to realise that there is no hard-and-fast line of demarcation between suspensoids and emulsoids; in the former the dispersed phase is relatively solid, while in the latter it is relatively liquid. Egg albumen and gum arabic in water are typical emulsoids; so also are many other common organic substances.

Now, in the emulsoids the distribution of the two phases can sometimes be reversed in response to chemical and physical stimuli. For example, cream is composed of oily globules dispersed in a watery fluid. Such an emulsoid,

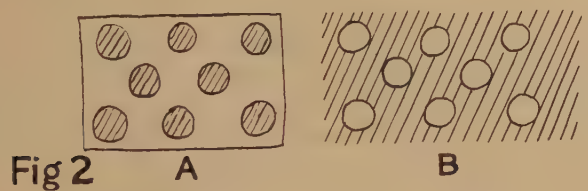


Fig. 2.—Reversal of Phases on a Colloid.

In A the watery phase is the continuous one, the oil phase (cross-hatched) being dispersed. This is the colloidal system of milk. In B the reversal of the phases has occurred—as in butter. The watery (and previously continuous) phase is now dispersed, while the oily (and previously dispersed) phase is now continuous.

which is termed a *Hydrosol*, is relatively fluid, because the more solid component is in a state of suspension in a fluid medium. When cream is churned a reversal of the phases takes place: the oily phase (previously dispersed) becomes continuous, enclosing the watery (and previously continuous) phase within it in the shape of globules. The fluid phase is now imprisoned within the alveoli of the more solid component. Consequently an emulsoid of this type is of a semisolid consistency, and is known as a *Hydrogel.* Fig. 2 illustrates this.

Again, gelatine in water is a truly reversible colloid. Beneath a certain temperature (*circa* 25° C.) it is a gel; when warmed it liquefies and becomes a sol; if it now be cooled

(under suitable conditions) it reverts to the gel condition. The whole reaction can be expressed thus :

$$\text{GEL} = \begin{cases} \text{Continuous (solid) phase} \\ \text{and dispersed (liquid) phase} \end{cases} \underset{\text{Below } 25^\circ \text{ C.}}{\overset{\text{Above } 25^\circ \text{ C.}}{\rightleftharpoons}} \text{SOL} = \begin{cases} \text{Continuous (liquid)} \\ \text{and dispersed} \\ \text{(solid) phases.} \end{cases}$$

Now protoplasm is colloidal and belongs to the group of emulsoids. This is now realised to be one of the basal conceptions of living matter. The microscopic study of live cells by special methods—together with much other evidence—shows it to be a colloid varying, through intermediates, from the sol to the gel condition.

The study of emulsoids—protoplasmic and other—with the dark-ground illumination, reveals them to be composed of innumerable shining particles, often in Brownian motion [1] in the hydrosol phase. These particles, unless of exceptional size, are microscopically invisible with ordinary direct illumination, the solution containing them appearing homogeneous. It is only when they are subjected to the intense oblique illumination of a dark-ground condenser (for an explanation and figure of which see p. 154) that they become visible, owing to the diffraction halo which is formed around every particle.

6. MICRO-DISSECTION

This is another means of attacking the problem of the intimate structure of protoplasm. As the name implies, it involves the dissection of living cells. This is done by means of very fine glass needles mounted in a special apparatus for manipulating them (Kite and Chambers).

The application of the methods of dark-ground illumination and micro-dissection have yielded important information regarding the living cell and its constituent elements. Details may be found in the original researches of Chambers, Bayliss,

[1] Brownian motion is the rapid dancing movement exhibited by very small particles in a fluid. It is due to the molecular bombardment of such particles.

and Kite, but the main facts resulting from them may be summarised as follows :

Observations with the dark-ground illumination on the protoplasm of amœbæ and leucocytes show that this is usually in the sol condition. During cell division and immediately preceding it, the protoplasm of these cells becomes a gel. A very weak electric current causes a transition from sol to gel, with ultimate return to the sol condition. The process is thus reversible. But if the current be somewhat stronger, the cell is killed and its protoplasm forms an irreversible gel. Some time after the onset of this, the sol condition may set in again. This, however, is a post-mortem liquefaction of the cell contents. And finally, if the current be relatively very strong, an amœba explodes, its protoplasm being rapidly dissolved in the surrounding water.

Some of the cells of vertebrates are in the gel condition when alive—*e.g.* nerve and muscle cells ; the protoplasm of embryonic germ cells and leucocytes appears to be a hydrosol, while gland cells may vary from sol to gel.

7. AUTOLYSIS

It is an established fact that tissues after death, unless kept at a very low temperature (*circa* 0° C.), become soft and ultimately liquefy after a varying time. This is not entirely due to the action of putrefaction bacteria, since this liquefaction, or *Autolysis*, occurs also when tissues which are bacteriologically sterile are aseptically kept in sealed glass tubes. The observations of Bayliss on Amœbæ seem to show that autolysis is a colloidal process involving the transition of the rigid gel state of recently dead protoplasm to a subsequent sol condition. It is essentially a liquefaction of living matter from hydrogel to hydrosol, and is due to the solvent action of intracellular ferments upon dead or dying tissues.

8. CHEMISTRY OF PROTOPLASM

Protoplasm is an extraordinarily complex mixture of proteids, fats, carbohydrates, and other substances. While the chemistry of living matter is altogether outside the scope of

this chapter, certain gross chemical differences in some of the cell components must be considered.

NUCLEUS

(i) **Nuclear Membrane.**—Regarding the chemical nature of this, nothing definite is known. The substance composing it is very variable in its staining reactions and has been termed "Amphipyrenin," which, however, is no more than a name, since it does not correspond to any definite chemical compound. All that can be said with certainty is that sometimes the nuclear membrane stains like the achromatic structures (*i.e.* the plasmosome and the so-called linin meshwork), sometimes like chromatin. This is probably due to the presence of minute granules of the latter substance being plastered on to the inner (nuclear) surface of the membrane.

(ii) **Chromatin.**—More is known about the chemical nature of chromatin than about any of the other nuclear structures. Chromatin resists dilute acids and both tryptic and (within measure) peptic digestion. But it is soluble in dilute alkalies. The resistance of chromatin towards peptic digestion is in contrast with the other nuclear elements, which are soluble therein, and it has enabled chromatin to be extracted from nuclei and then analysed. In this way it has been found that chromatin principally consists of nucleic acid—which is characterised by its high phosphorus content of 8 to 10 per cent.—and albumen. There is evidence to show that the proportions of these two compounds vary greatly in relation to the physiology of the cell. Thus, during mitosis the nucleic acid content of the chromosomes is probably far higher than in the chromatin of the resting cell.

(iii) **Nucleolus.**—In the case of chromatic nucleoli or *karyosomes*, their composition is identical with that of chromatin, since they are merely condensations of the latter. *Plasmosomes*, or achromatic nucleoli, give very different microchemical reactions, for while they resist dilute acids (like chromatin), they are attacked (in contrast to it) by peptic digestion under suitable conditions. Further, plasmosomes and karyosomes behave differently towards dyes. The sub-

stance composing achromatic nucleoli has been called "Pyrenin," but nothing definite is known about this material chemically, beyond that it is a nucleo-albumin.

(iv) **Karyolymph.**—The nuclear sap, as already explained, is the fluid within the nuclear membrane. It probably contains albuminous substances in solution.

CYTOPLASM

(i) **The Centrosome.**—This body resists even highly acid solutions and stains with great intensity with certain dyes, especially iron-hæmatoxylin. Regarding its true chemical nature, nothing is known.

(ii) **The Golgi Bodies.**—According to Cajal the Golgi elements contain a mixture of proteids and fats as judged by their histo-chemical reactions. There can be little doubt but that the chemical constitution of the Golgi bodies varies considerably in relation to the activity of the cell.

(iii) **The Mitochondria.**—Chemically these are a mixture of phospho-lipin and albumen in varying proportions. They are more or less soluble in dilute acetic acid, highly so in alcohol, chloroform, and ether. Under the influence of moderate heat (48° to 50° C.) they dissolve in the cytoplasm of unfixed cells, in contrast with the nuclear elements. Since mitochondria are lipoidal bodies and not true fats they do not give the microchemical reactions of the latter.

(iv) **The Metaplasmic Inclusions** comprise many substances, of which fat, glycogen, and yolk are the most important.

The *Fats* of vertebrates are chiefly derivatives of oleic, stearic, and palmitic acids, the former usually unsaturated, the two latter saturated. The tissue fats are mixtures of both saturated and unsaturated fats.

Glycogen is a carbohydrate with the empirical formula of $(C_6H_{10}O_5)n$. It is water-soluble, but can be precipitated by absolute alcohol.

Yolk has been carefully analysed in the bird's egg, where it is found to be a complex chemical mixture of protein, fats, lecithin, and cholesterin. Yolk is chemically one of the most variable of the cell constituents, not only because it is a mixture

of so many substances, but because the relative proportions of these vary at different stages of the development of the egg cell. In fact, the histo-chemical reactions of yolk are governed chiefly by the substance which is present in the highest proportion ; when this is fat, the yolk behaves like fat towards reagents, when this is lecithin it reacts like this substance.

To summarise the foregoing paragraphs, it may be said that the nuclear components are more resistant towards reagents than the cytoplasmic ones. Chromatin is particularly resistant. The cytoplasmic elements, on the other hand, are easily dissolved out during fixation. This is because they are usually lipoidal, fatty, or starchy in nature.

Regarding the true chemistry of these bodies, with the exception of chromatin, our knowledge is very scanty. Usually all that is possible is to relegate a given cell structure to one of the great chemical groups—*i.e.* we say that it is a "fat," a "lipoid," or "carbohydrate," and so on. The fact that the various cell components are mixtures of organic compounds of the most complex molecular structure renders their microchemistry very difficult. The molecular weight of hæmoglobin, for instance, is generally regarded as being 16,669 ! And this difficulty is enhanced by the small size of the elements of the cell and the frequent impossibility of extracting any one of them without extracting others at the same time. Unless new methods are evolved, it is difficult to see how our vague and fragmentary knowledge of the chemistry of the cell components can be improved.

CHAPTER III

OUTLINE OF METHODS EMPLOYED IN HISTOLOGY

1. Examination of Fresh Cells and Tissues; Dissociation—2. The Smear Technique—3. The Sectional Method—4. Intravitam Staining—5. Dark-Ground Illumination.

Histological technique comprises the methods by which cells and tissues are prepared for examination with the microscope. For this purpose one may proceed along one or more of the following lines :

1. EXAMINATION OF FRESH CELLS OR TISSUES ; DISSOCIATION

In the case of cells which are suspended in a fluid—*e.g.* blood or lymph—this may be done by *direct examination* of a drop of the fluid.

In the case of cells which are grouped together to form a solid mass, whether a tissue (*e.g.* subcutaneous connective tissue) or an organ (*e.g.* the liver), it is necessary to separate the cells from one another in some indifferent fluid medium, in order to obtain a sufficiently thin layer of cells to study with the microscope. This process is known as *dissociation* or *teasing*.

2. THE SMEAR TECHNIQUE

This is a derivative of the above. By it the constituent cells of organs or tissues are mechanically smeared upon a microscope slide or cover-slip. They are then *fixed* in order to preserve their appearance in as lifelike a manner as possible. Next, the cell or tissue components are *stained* so as clearly to bring into evidence their constituent elements. And finally

they are *mounted* in some medium such as Canada balsam, which imparts a suitable degree of optical refractility to the tissue.

3. THE SECTIONAL METHOD

This involves the cutting of tissues into very thin slices or *sections*. This process has the advantage—in contrast to (1) and (2)—that the relations of the cells to one another in an organ are not disturbed. Even minute details, such as the passage of a white blood corpuscle through the wall of a capillary blood vessel, are accurately preserved. Obviously, however, the interpretation of sections demands experience. The mere fact that tissues are being studied in such thin slices (usually of 5 to 10 μ [1] in thickness) gives them a peculiar appearance. But with practice the interpretation of such images becomes easy, while the accuracy of the method is such that nearly all research and routine work in histology is accomplished by one of the sectional methods.

To prepare tissues for sectioning there are three methods in common use : the *Paraffin*, the *Celloidin*, and the *Freezing* methods. In the first, the tissues are permeated with molten paraffin, which is then allowed to solidify by cooling. In the second, the object is impregnated with solutions of increasing strength of celloidin (a purified form of gun-cotton). Finally, the celloidin is caused to " set "—usually by exposure to chloroform or its vapours.

With either of these methods the end result is the same : the tissue is permeated by a medium of such a consistency as to enable thin sections to be cut, attached to slides, stained, and mounted. The object is then ready for examination with the microscope.

In the third—the freezing—method, the tissues are frozen to a suitable degree of consistency. They are then sectioned. Sometimes they are permeated, before being frozen, with gelatine or gum arabic. The tissues are finally stained and mounted.

[1] The micron (usually abbreviated to μ) is the standard unit of measurement in microscopy. $1\,\mu = \frac{1}{1000}$ of a millimetre, *i.e.* $\frac{1}{25{,}000}$ of an inch.

4. THE METHOD OF INTRAVITAM STAINING

This is the method by which living cells are stained, usually after dissociation in the staining fluid. This technique has not as yet become a part of general histological technique, though as a control to cytological studies on fixed and stained material it has proved of great value.

5. THE DARK-GROUND ILLUMINATION

This method, usually applied to fresh tissues, is used for the detection of micro-organisms in body fluids—*e.g.* Trypanosomes in blood, or the *Spirochæta pallida* in chancre exudate. For the latter purpose it is in use in every venereal clinic, and is hence a method of particular importance. Quite recently much has been learned about the colloidal nature of protoplasm by the application of this method to large cells such as leucocytes or amœbæ. The use of the dark-ground technique, particularly for the study of protoplasm, demands considerable experience in the interpretation of the images furnished by it. Even in the hands of the expert, knowledge of the material as seen with direct illumination is necessary if one is not to be misled by the peculiar appearances (see p. 155) inherent to the optical arrangement of the dark ground.

PART II

TYPE METHODS OF NORMAL HISTOLOGY

CHAPTER IV

FIXATION

1. Aim of Fixation—2. Effects of Fixation—3. Artefact and Fixation—4. Some Common Substances employed in Fixatives—5. Choice of a Fixative—6. Type Fixatives containing the Substances already described: A. Micro-Anatomical; B. Cytological (Nuclear and Cytoplasmic)—7. Tonicity of Fixatives—8. Post-mortem Changes in Tissues—9. Incompatible Fixatives and Stains—10. General Precautions.

1. AIM OF FIXATION

The aim of fixation is to preserve the cell and tissue constituents in as lifelike a condition as possible. At the best, the cell and tissue elements are preserved so that they *look* like what they were during life, though the mere fact that they are no longer living is in itself proof that what we see with the microscope is no longer protoplasm. In fact, the fixed cell is to protoplasm what the embalmed body is to a living man—a more or less accurate imitation.

In addition to the preservation of the components of tissues, it is necessary that the fixative employed should inhibit post-mortem changes whether putrefactive or autolytic. Obviously once these changes have set in, the tissues have ceased to represent the living condition. Hence the need for tissues to be as fresh as possible.

2. EFFECTS OF FIXATION

The chief effect of fixation is to render cell and tissue constituents resistant to such subsequent treatment as they may have to undergo before being examined with the microscope.

And this is attained by their being coagulated or precipitated by the fixative, and hence made insoluble.

Some histologists distinguish between precipitation and coagulation. Precipitation is said to be a physical, coagulation a chemical, change.

By *precipitation* they imply the throwing down of protoplasmic substances by the fixative without any chemical combination occurring between them and the precipitating agent. Absolute alcohol, for example, is a fixative of this type. It " fixes " glycogen simply because it throws it down from a pre-existing state of watery solution.

Coagulation, in contrast, is regarded as being of a chemical nature. Reagents which fix protoplasmic substances in this way do so by combining chemically with them. Thus, picric acid forms compounds with the cell proteids.

Note that this distinction while being of theoretical interest is of little practical value. For many reagents both precipitate and coagulate, in addition to which, further complexity of action is caused by fixing fluids being nearly always mixtures of several substances.

Fixation, however, has various accessory effects which are sometimes very important.

(i) Some fixatives act as *mordants for certain stains*. For present purposes this action may be explained by saying that the fixative makes possible, or facilitates, a particular staining reaction. Thus, the best results when tissues are stained with safranin are obtained after they have been fixed in Flemming's fluid.

(ii) Again, fixatives usually have a *hardening effect on tissues*, although this effect has usually to be reinforced by the action of strong alcohol before tissues are of a consistency sufficient to allow of their being cut into sections.

(iii) Another effect of fixation is to *render cells insensitive to hypo- and hyper-tonic solutions*.

(iv) And finally, *fixatives usually aid the optical differentiation* of cell and tissue constituents. The refractive index of these is raised in varying degrees. This is very important, because certain of the elements of the cell are of a refractive index so akin to that of the surrounding substance (*e.g.* ground cytoplasm) as to be almost—or completely—invisible in the living cell. Once fixed, however, they can often be differentiated from one another even when unstained.

3. ARTEFACT AND FIXATION

We have seen that the fixed cell can never be more than the simulacrum of the living one. The question now arises as to whether even this imitation really looks like what one can see in the living cell, or, if not, in what respects it differs from it. For even if the image is not quite the same, it will yet yield valuable information provided that it is possible to discriminate between what is real and what has been added to (or taken away from) the picture of protoplasm by the process of fixation.

In this connection it is of paramount importance to bear in mind that *living protoplasm is a mixture of colloids of a fluid or semi-fluid consistency.*

Now the problem of fixation can only be rationally discussed by taking into consideration the above.

Colloids and Fixation.—What is the effect of fixation upon a colloidal substance—protoplasmic or otherwise ? In so far as the gross effects are concerned the answer is obvious : the fluid or semifluid condition of living matter is destroyed by fixation and subsequent treatment. It is rendered solid, and were this not so, it would be impossible to cut sections of it as we do.

Investigation, however, has made further progress than this. Hardy's valuable researches on the action of fixation on the optical appearances of artificial colloids and animal cells may be briefly summarised as follows :

A 13 per cent. solution of gelatine in water when allowed to cool becomes a hydrogel of alveolar structure, as already described. The droplets of water are definitely bounded by the gel component to such an extent, indeed, that even a pressure of 400 lb. per square inch does not suffice to squeeze them out.

Now, if gelatine, when set, is fixed in formol, its structure is changed. It is no longer alveolar but sponge-like, while the reticulum formed by the gel component does not hold the water as isolated droplets. In fact, the latter can easily be squeezed out by hand.

If the gelatine be fixed in absolute alcohol or in a saturated

solution of mercuric chloride ($HgCl_2$) in 0·6 per cent. saline, a similar meshwork is formed. And finally, if the concentration of the gelatine be altered, differences in the size and character of the meshes are produced.

Hardy also studied the effect of fixation on animal cells. He found that the character of the ground cytoplasm varied with different fixatives. Thus, intestinal epithelial cells of *Oniscus* (a freshwater crustacean) show a fine cytoplasmic network when fixed in either osmic acid vapour or corrosive sublimate. But the network differs in the two cases.

Further, recent research on the protoplasm of living cells rarely reveals any special structure beyond such as displayed by other colloids of the emulsoid type. The protoplasm of the cells of vertebrates, as already pointed out, is seen to consist of innumerable colloidal particles, and is sometimes in the sol, sometimes in the gel, condition, when studied with the microscope. Beyond this, protoplasm is structureless, and, when studied by ordinary methods of microscopic examination, appears to be homogeneous. And when fixed, stained, and cut into very thin sections it seems to be of the nature of a meshwork, an appearance which all recent evidence points out to be an artefact due to fixation.

Note that this statement only concerns the intimate structure of protoplasm. It does not apply to the general appearance of the cell components—*e.g.* nucleoli, mitochondria, fat globules, etc. For these bear no more relation to the fundamental structure of protoplasm than do the shape of the bricks to the intimate composition of a wall.

Summarily expressed, the main effect of fixation on protoplasm is to cause it to "set" into the condition of a rigid and irreversible gel. This process is accompanied by visible changes, chief of which is the formation of an artificial network (only visible in very thin sections) in what were the more fluid portions of the cell during life. This network is specially visible in the ground cytoplasm after fixation. Only rarely—as possibly in the Echinoderm egg —may the alveolar theory of Bütschli have its counterpart in the living cell.

4. SOME COMMON SUBSTANCES EMPLOYED IN FIXATIVES

Beneath is given a list of some of the more important fixing reagents. Usually several of these substances are combined in a given fixative. This is because no single reagent has the power of fixing all the cell and tissue components. So an attempt is made to preserve as many of these as possible by combining several reagents together. In so doing, however, one has to be careful that the components of a fixative are not chemically of such a nature as to combine *inter se* to form different compounds. Thus, mixtures of oxidising and reducing agents, or of acids and bases, should not generally enter into the composition of any fixative.

Stock solutions of the following substances should be kept. The amount of stock solution to be made up is obviously in relation to the amount of fixing done in the laboratory. But it is usually sufficient to keep 1-litre bottles of these reagents, with the exception of osmic acid, which is costly, only used in small quantities, and very apt to deteriorate on keeping. Therefore 50 or 100 c.c. of a 2 per cent. solution of this reagent is an ample stock solution for most laboratories.

Formaldehyde (H.CHO).—Formaldehyde is a gas which is soluble in water to the extent of about 40 per cent. by weight, and is sold in such a solution under the name of *Formol* or *Formalin*.[1] This solution goes acid on keeping, owing to the production of formic acid, but can easily be neutralised by adding to it either a 40 per cent. solution of caustic soda drop by drop, or by simply letting it stand for some hours over calcium carbonate. Commercial (*i.e.* 40 per cent.) formalin often becomes turbid—especially if kept in a very cold place —through the formation of a white precipitate of paraformaldehyde.

Pure (40 per cent.) formalin is unsatisfactory as a fixative ; it is usually employed in aqueous or saline solutions varying from 3 per cent. to 10 per cent. of formaldehyde. In making up these solutions it is important to remember that the strength

[1] *Throughout this book the percentage of formalin is referred to in terms of the percentage of formaldehyde : thus,* 20 *per cent. Formol = equal parts of commercial formalin and water.*

of the commercial product is 40 per cent.—*not* 100 per cent. The following table indicates the proportions of commercial formalin and water (or saline) required for making up the lower concentrations :

Grade Required.	Volumes of 40 per cent. Formol.	Volumes of Water.
3 per cent.	7·5	92·5
5 ,,	12·5	87·5
10 ,,	25·0	75·0
20 ,,	50·0	50·0

Formalin vapour is injurious to the eyes and nasal mucosa, and should be avoided as far as possible. Unpleasant, also, is its effect upon the hands, which should be protected by rubber gloves when handling formalin-preserved specimens.

Formalin does not form salts, but additive compounds with cell and tissue constituents. It preserves fats in the sense that it does not dissolve them, but it in no wise renders them resistant to subsequent treatment with fat solvents.

Mercuric Chloride (= Mercury Perchloride; Corrosive Sublimate) ($HgCl_2$).—Mercuric chloride is sold in the form of heavy white crystals. It is soluble in water to about 7 per cent. at room temperature—*i.e.* 18° to 20° C. In boiling water its solubility is about 53 per cent., in cold alcohol 33 per cent. This salt combines with the cell proteids to form albuminates of mercury.

Osmium Tetroxide (= Osmic Acid) (OsO_4).—This very costly reagent is supplied in sealed glass tubes of 0·5 or 1 gramme. In making up fixatives it is employed in aqueous solutions of 0·5 per cent. to 2 per cent. A stock solution of the latter should be kept. The latter is a nearly saturated solution and takes several days to dissolve. In preparing solutions of osmic acid proceed thus: Remove the label and all traces of gum from the tube with water. Dry, file, and break it in the middle. Then throw the two halves into the proper quantity of distilled water in a glass-stoppered bottle. Keep in a cool place. Remember that solutions of

osmic acid are very easily spoilt by reduction with the formation of a black precipitate representing a lower oxide of the metal. To avoid this, use pure and dust-free distilled water, and never warm in order to dissolve the crystals quicker. Even moderate warmth, or a trace of organic matter, suffices to reduce the solution. Should this occur, in spite of precautions, the solution can sometimes be regenerated by adding a few drops of either 10 per cent. chromic acid or a 10-volume solution of hydrogen peroxide. Never pour used osmic acid back into the stock bottle. It is a false economy, as it is liable to reduce the stock solution.

Both crystals and solution give off a particularly irritating vapour, liable to cause conjunctivitis.

OsO_4 combines with the cell proteids to form additive compounds. The black staining of fatty substances by it has recently been shown by Partington and Huntingford to be due to the formation of a hydrated form of *osmium dioxide*, probably $OsO_2.5H_2O$.

Chromic Acid (H_2CrO_4) is prepared by dissolving the dark red crystals of the anhydride (CrO_3) in distilled water. A 2 per cent. solution is the one most usually employed in making up fixatives, though a stronger one (10 per cent.) may be kept as a stock solution, and the weaker grades made from it as occasion arises.

Chromic acid combines with proteids to form insoluble compounds.

Potassium Bichromate ($K_2Cr_2O_7$) is one of the oldest and commonest fixing reagents. The usual solution is one of 3 per cent. in distilled water. A saturated aqueous solution contains about 12 per cent. of the salt at room temperature.

Potassium bichromate forms insoluble compounds with the cell proteids and lipoidal bodies—*e.g.* mitochondria.

Picric Acid ($C_6H_2(NO_2)_3OH$).—This is usually kept as a saturated aqueous solution. Crystals are put into a large bottle of distilled water, and fresh water is added every time some of the solution is poured off. The solubility of picric acid is between 0·5 per cent. and 1 per cent. at room temperature.

Picric acid is a powerful coagulant of protoplasm, combining

with the cell proteids to form (it is alleged) water-soluble picrates.

Acetic Acid ($CH_3.CO.OH$).—When pure, this is called "glacial" acetic acid, because it becomes solid at 17° C. A varying amount of glacial acetic acid enters into the composition of many fixatives.

While it is an energetic precipitant of chromatin, it has a solvent action on many of the cytoplasmic bodies—*e.g.* mitochondria and Golgi bodies.

Alcohol (*i.e.* ethyl alcohol, C_2H_5OH) precipitates proteids. When used alone, absolute (100 per cent.) alcohol should be taken. It causes much shrinkage, and attacks mitochondria and fat, but preserves glycogen. Chiefly used for histochemical purposes.

Ether (*i.e.* ethyl ether ($(C_2H_5)_2O$) and Chloroform ($CHCl_3$) are energetic fat and lipoid solvents. They therefore have a very destructive effect on the cytoplasmic constituents.

All of the "type" fixatives described in the following sections contain one or more of the above substances.

5. CHOICE OF A FIXATIVE

When one fixes protoplasm it is necessary to determine whether the particular object in view is the preservation of tissues (*i.e.* gross structure) or of the constituent elements of the cells themselves (*i.e.* very minute structure). The technical limitations are such that it is nearly always impossible to do both simultaneously. It is as difficult—even more difficult—to kill two birds with one stone in histological technique as in any other branch of human endeavour.

Fixatives may be divided into two main groups according to their uses.

(i) **Micro-Anatomical Fixatives**, which aim at accurately preserving the relations of tissue layers and large aggregates of cells to one another. Most of the routine work of normal and pathological histology is done with such fixatives.

(ii) **Cytological Fixatives**, which aim at preserving the constituent elements of the cells themselves. Penetrative power, the ability to work with large masses of tissue, ease

in subsequent manipulation and staining—essentials from the micro-anatomical point of view—are sacrificed towards this end.

As in any system of arbitrary classification, there is a certain amount of overlap in grouping fixatives in this way. But this does not matter so long as it is realised that certain micro-anatomical fixatives can sometimes be used cytologically and *vice versa*.

6. TYPE FIXATIVES CONTAINING THE SUBSTANCES ALREADY DESCRIBED

The different formulæ for fixatives are legion. Fixatives are usually named after their inventors. Many of them are only trivial and useless modifications of some pre-existing fluid, and their chief claim for existence appears to be that they perpetuate their inventor's name.

In accordance with the scheme of this book only a very limited number of type fixatives are given. But once the technique of these has been mastered, proficiency with other methods will be easy. The more special methods of fixation are included in Part IV. (Methods for Special Organs).

A. MICRO-ANATOMICAL FIXATIVES

Bouin's Fluid.—

Picric acid, saturated aqueous solution . .	75 c.c.
40 per cent. formol	25 c.c.
Glacial acetic acid	5 c.c.

This solution keeps indefinitely. Drop into it pieces of tissue —preferably not more than 1 cm. in thickness. Leave therein for one-half to twelve hours according to thickness and consistency. Wash out in 50 per cent. alcohol, *not* water, in order to avoid the possibility of dissolving out some of the water-soluble compounds formed between picric acid and the cell proteids.

This fixative usually causes but little shrinkage of tissue layers. Should it do so in some cases—*e.g.* embryos—dilute it with 25 to 50 per cent. of its volume of distilled water. The exact degree of dilution has to be ascertained by experiment. The picric acid stains the tissues yellow ; this is generally an advantage—especially when one is dealing with small objects

—because the sections are easily seen during section cutting, while the yellow colour can be subsequently extracted from the sections on the slide.[1]

Bouin's fluid gives excellent micro-anatomical pictures of all mammalian tissues with the curious exception of kidney, which is atrociously preserved by it.

Owing to its acetic acid content, Bouin is unreliable for cytological observations on the cytoplasm, inclusions such as Golgi elements and mitochondria being either distorted or actually dissolved. For work on the cytology of the nucleus Bouin's fluid is often of value in studying chromosomes (counts, etc.) or chromatin.

ZENKER'S FLUID.—

Mercuric chloride. . .	5 grms.
Potassium bichromate . .	2·5 grms.
Sodium sulphate . . .	1 grm.
Aq. dest.	100 c.c.
Glacial acetic acid . .	5 c.c.

Zenker's fluid keeps badly. This may be avoided by preparing a stock solution of it *minus* the acetic acid, which is only added immediately before using it.

Fix tissues three to twelve hours. Wash them overnight in a capsule under a gentle stream of tap water,so as to remove any surplus potassium bichromate. Transfer them first to 50 per cent. and then to 70 per cent. alcohol (several hours at least in each), to which sufficient iodine has been added to make the alcohol dark brown. The simplest way is to keep a saturated solution of iodine in 90 per cent. alcohol in a drop-bottle. If the alcohol becomes clear after adding the iodine solution (which it may do after several hours), add more iodine, and go on doing so until the alcohol remains brown. This indicates that all free corrosive sublimate has been dissolved in the iodine-alcohol. It is important that this precipitate of mercuric chloride should be removed, since if there be much of it, it is a hindrance to subsequent microscopic observation.

NOTE.—The $HgCl_2$ deposit can be removed from the sections on the slide, and the iodine treatment during dehydration omitted. (See p. 124.)

[1] By placing the sections in a saturated solution of lithium carbonate in 70 per cent. alcohol for two to five minutes.

ZENKER-FORMOL.—

Omit the acetic acid from the above.
Substitute 5 c.c. of 40 per cent. formol immediately before use.

In spite of its irrational formula—potassium bichromate and formol, oxidising and reducing agents respectively—this is an admirable general fixative for mammalian tissues. Fixation and after-treatment, as for Zenker, except that the duration of fixation is best prolonged to twelve or twenty-four hours.

From the point of view of gross structure Zenker is an efficient fixative and is easy to work with. Some of the American pathologists employ it exclusively. Its disadvantage is that first the potassium bichromate and then the mercury salt need careful washing out.

FORMALDEHYDE is probably the most commonly employed fixing reagent. The various strengths can be made up according to the table at the beginning of the book, but the usual one is prepared by taking one volume of commercial (40 per cent.) formalin to eight or nine volumes of isotonic (0·9 per cent.) saline. This gives a strength of 4 to 5 per cent. It is very widely used for fixing pathological material.

Specimens are left in this fixative for twenty-four to forty-eight hours, and are then transferred to 50 per cent. alcohol. But they may be left in for much longer (even months) as formol not only fixes but also preserves. When used for the latter purpose it should be changed at least once.

MERCURIC CHLORIDE is generally employed in *saturated aqueous solutions*. It penetrates and fixes very rapidly, so rapidly as often to cause much cell shrinkage when employed alone.

Very small pieces of tissue are thrown into the fixative and taken out as soon as they have become opaque—*i.e.* after a few minutes. Larger objects are fixed for several hours. Tissues are then treated with 50 per cent. and 70 per cent. iodine-alcohol as explained on p. 36.

Probably the most useful formula for a mercury-containing fixative is :

$HgCl_2$ saturated aqueous solution . . .	90 c.c.
40 per cent. formalin (commercial) . . .	10 c.c.

Fix for one to twelve hours. Then treat with alcohol and iodine. This solution has the advantage of not only preserving tissue layers with the minimum of distortion, but also of retaining much of the finer (cytological) detail of the cells themselves. Red and white blood corpuscles are well preserved. I use this fluid for much routine work.

Absolute Alcohol is chiefly used for histo-chemical purposes, for which it is important that it should be pure. Tissues are thrown into several changes of this, and are then cleared and embedded. If left for longer than forty-eight hours in absolute alcohol, most tissues become so hard as to render section cutting impossible. As an ordinary micro-anatomical fixative, absolute alcohol is not good, since it usually causes much shrinkage. Its chief use in histology is as a preservative for glycogen. Delicate cytoplasmic inclusions, such as fat, mitochondria, and Golgi bodies, are destroyed by it.

B. CYTOLOGICAL FIXATIVES

It is convenient to divide cytological fixatives into those suitable for work (i) on the nucleus, and (ii) on the cytoplasm and its inclusions. Under (i) come chromatin network and chromosomes, and (to a lesser extent because they are preserved by any technique) the nucleoli. Under (ii) come the mitochondria, Golgi bodies, fat, yolk, glycogen, and various secretory products.

(i) Nuclear Fixatives

Flemming's Fluid.—

1 per cent. chromic acid	15 c.c.
2 per cent. osmic acid	4 c.c.
Glacial acetic acid	1 c.c.

This fixative keeps badly. The stock solution should therefore be small. It penetrates poorly, consequently pieces of tissue should be thin (not more than several millimetres in thickness). Fixation should last from one to twenty-four hours ; twelve hours is the usual time. Wash the tissues overnight in running water and transfer to 50 per cent. alcohol.

The bulk of this fixative need only be five to ten times that of the object. Do not pour used Flemming back into the stock bottle.

Flemming's fluid used to be indiscriminately employed for all cytological work. It is now recognised that its chief use is for fixing nuclei, and especially chromosomes. As shown by Gatenby, ordinary Flemming gives very deceiving pictures of the cytoplasm, since it contains 5 per cent. of acetic acid, which is quite sufficient to distort or destroy certain cytoplasmic inclusions such as Golgi bodies or mitochondria.

Bouin's Fluid is sometimes useful for chromosome work. It penetrates far better than Flemming, but is more destructive to cells. The way to use Bouin has been described on p. 35.

Carnoy's Fluid.—

Absolute alcohol	60 c.c.
Chloroform	30 c.c.
Glacial acetic acid	10 c.c.

Fix tissues for ten minutes to one hour. Wash out the acetic acid in several changes of 90 per cent. alcohol. Carnoy is one of the most penetrating fixatives known. It is much used for work on chromosomes, the number of which can often be more easily ascertained by using this fixative than any other. This is probably because of its solvent action on the elements of the cell other than chromatin. The chromosomes consequently stand out more or less naked in the surrounding cytoplasm. Since the three constituent substances of Carnoy (alcohol, chloroform, and acetic acid) are powerful lipoid and fat solvents, this fixative is useless for any work on the cytoplasm. Of the elements of the latter, only glycogen (which is precipitated by the alcohol) and the centrosome resist it.

(ii) Cytoplasmic Fixatives

To fix the cytoplasm and its inclusions in a trustworthy manner requires special and careful technique. Only a few standard methods are given in the ensuing section. For further information see the admirable chapter by Gatenby on "Cytological Methods" in the *Microtomist's Vade Mecum* (p. 292).

Flemming without Acetic (Gatenby's Modification).—This is prepared in the same way as ordinary Flemming, except that the acetic acid is omitted. It has the advantage over the standard solution in that it keeps well. Pieces of tissue,

which must be very thin, are fixed for twelve to twenty-four hours. They are then washed in running water and transferred to 50 per cent. alcohol. As with all chrome-osmium mixtures, penetration is poor. The outermost layers of the object are overfixed and often blackened by excessive reduction of the osmium. The cells slightly farther in are properly fixed. The innermost cells are usually macerated and stain very badly—unless the object be exceptionally thin. Critical work has therefore to be restricted to the intermediate portions of the object. With some tissues this solution causes shrinkage. Correct this by adding one-half to one-third of distilled water to the volume of the fixative.

CHAMPY'S FLUID.—

3 per cent. potassium bichromate . . .	7 c.c.
1 per cent. chromic acid.	7 c.c.
2 per cent. osmic acid	4 c.c.

The results obtained by this fixative are very similar to those given by Flemming without acetic. Use in exactly the same way.

The methods of Gatenby and Champy preserve mitochondria, fat, and yolk. They often give better results with the tissues of invertebrates and lower vertebrates than with those of mammals.

REGAUD'S FORMOL BICHROMATE.—

3 per cent. potassium bichromate . . .	80 c.c.
40 per cent. (commercial) formalin . . .	20 c.c.

The solution is unstable and should be made up immediately before use. Fix tissues in this for four days, *changing the fluid every day*. Wash in running water twenty-four hours, and transfer to 50 per cent. alcohol, or else submit the tissues to the process of post-chromatisation (see p. 43).

Regaud gives excellent results with mammalian tissues. It is less costly and penetrates better than the chrome-osmium fixatives. Mitochondria and yolk are preserved by it, but not fat.

7. TONICITY OF FIXATIVES

The relation between the osmotic pressure of a fixative and the degree of cell shrinkage or swelling is not yet clear. Solutions of formol, however, should preferably be made up in

isotonic saline and *not* in water, while saturated solutions of mercuric chloride may be dissolved in *either* water *or* saline. It has been shown (Carleton) that a 4 per cent. solution of formol, if made up in distilled water, gives rise to vacuolation and swelling of the cytoplasm of cat and frog tissues. No difference could be detected in the same tissues fixed in saturated (6 per cent.) solutions of mercuric chloride dissolved in distilled water and isotonic saline.

8. POST-MORTEM CHANGES IN TISSUES

Putrefaction and autolysis not only distort or destroy the finer structures of cells and tissues, but also upset their staining reactions.

The sensitivity of different organs towards post-mortem changes varies. Thus, the alimentary canal is the first to suffer, while muscle, and notably cardiac muscle, one of the last. Blackwood has shown (i) that post-mortem changes proceed most rapidly during the first twenty-four hours subsequent to death, after which they become progressively slower ;[1] (ii) that the fundamental change is one of shrinkage, beginning with retraction of the cytoplasm from the cell membrane and ending with the desquamation and final disintegration of entire masses of tissue. The epithelia of all the tubular structures is especially liable to these changes ; (iii) the nuclear changes are subsequent to the cytoplasmic ones ; (iv) better results are obtained as regards preservation and staining of post-mortem material by the use of fixatives containing mercuric chloride than with formol alone.

Post-mortem changes are most rapid in their onset in tissues which normally contain putrefactive bacteria—*e.g.* the intestine. The green or yellow discoloration of tissues adjacent to the alimentary tract—notably the liver and skin of the abdomen—is due to the action of hydrogen sulphide from the alimentary canal on the hæmoglobin in these tissues. The central nervous system is also very sensitive to autolytic changes.

[1] This remark applies to temperatures between 50° and 60° F. (10° and 15° C.). I have to thank Miss Blackwood for allowing me to summarise work as yet unpublished.

9. INCOMPATIBLE FIXATIVES AND STAINS

Many fixatives do not permit of staining with all stains. As already mentioned, some fixing reagents act as mordants for particular staining processes. Inversely, a fixative sometimes inhibits certain stains. And since fixation is the first step in histological technique, and staining is always subsequent to it, it is necessary in the choice of a fixative to bear in mind any limitations which it may impose upon staining.

The behaviour of the fixatives already described towards stains is indicated in the following table :

Bouin
Sublimate-formol
Mercuric chloride
Carnoy
} allow of most methods of staining.

Zenker also allows of most methods of staining—provided it be properly washed out of the tissues after fixation.

Absolute alcohol : tissues stain feebly with dyes other than carmine ones, which do not act at all.

Formol (*i.e.* various dilutions of formol in water) : staining with the different varieties of hæmatoxylin is intensified. It inhibits the carmine stains, and, to a limited extent, the acid aniline dyes—*e.g.* eosin.

Regaud : poor with carmine stains. Best results with hæmatoxylin of Heidenhain or Delafield. Ehrlich's hæmatoxylin stains fairly well, but the staining time has to be lengthened.

Chrome-osmium [1] inhibits carmine dyes and Ehrlich's hæmatoxylin. But by staining overnight (instead of five to ten minutes) with the latter it is sometimes possible to get satisfactory results. Works well with iron-hæmatoxylin (long method). Acts as a mordant for the basic aniline dyes, and especially safranin.

10. GENERAL PRECAUTIONS

Remember to eliminate *mercuric chloride* precipitate from tissues before staining. This may be done either during

[1] Chrome-osmium fixatives are those which contain a chrome salt or chromic acid) plus osmic acid.

dehydration (see p. 36) in the case of small pieces of tissue, or when sections are on the slide (see p. 124).

Chrome-osmium tissues need at least twelve hours washing in running water. The same applies to fixatives containing potassium bichromate.

Wash out *formol* and *Bouin* tissues in 50 per cent. alcohol, *not* water.

When using the basic aniline dyes, and especially safranin, with Bouin-fixed material, all trace of picric acid must be removed from the sections before staining. Unless this be done, precipitates form.

To preserve tissues prior to embedding them, see p. 47.

The bulk of the fixative should always be fifty to a hundred times as much as the bulk of the object, with the exception of the chrome-osmium fixatives. With the latter five to ten times as much of the fixative suffices.

Note that (as said by Langeron) *good fixation is the cornerstone of all good histology.* Poorly fixed tissues are always open to faulty interpretation. No matter how good subsequent technique may be, a badly preserved object remains irretrievably bad, and as such is thoroughly untrustworthy for any criticial work.

The freshness of tissues is important, and for all cytological work an essential. For the latter purpose tissues should be removed from the body immediately after death. Failing this, organs may be kept at +2° to +6° C. in the ice chest for many days without damage.

Post-Chromatisation.—By this is meant the treatment of tissues which have been previously fixed in formol-bichromate or Flemming without acetic acid acid with a solution of potassium bichromate. It is especially used to mordant mitochondria.

Fix in the ordinary way ; transfer the tissues direct to a $2\frac{1}{2}$ to 3 per cent. solution of potassium bichromate ; leave therein for three to seven days. Then wash the material for twelve to twenty-four hours in running water.

CHAPTER V

DEHYDRATION, CLEARING, AND EMBEDDING

1. Introductory—2. Dehydration—3. Preservation of Tissues—4. Identification of Tissues—5. Paraffin Embedding (Clearing; Impregnation; Orientation; Casting)—6. Vacuum (Paraffin) Embedding—7. Faults in Dehydration, Clearing, and Embedding—8. Scheme of Processes involved in the Preparation of Tissues for Sectioning by the Paraffin Method—9. Celloidin Embedding ("Dry" and "Wet" Processes)—10. Double Embedding.

1. INTRODUCTORY

THE sectional method is the standard way of preparing tissues for examination with the microscope. But before they can be sectioned it is necessary to submit them to certain preparatory treatment. The latter usually involves the inclusion of the object in a mass of such consistency as will enable thin sections to be cut. This process is termed "inclusion" or "embedding."

Sectional methods may be grouped under three headings:

The Paraffin Method, by which the object is permeated with, and encased in, molten paraffin wax, which is then allowed to solidify by cooling.

The Celloidin Method, involving the inclusion of the object in a solution of celloidin (a purified form of gun-cotton), which is then caused to set and harden.

The Freezing Technique, by means of which the tissues—often after preparatory soaking in a solution of gum arabic or gelatine—are placed on the object-holder of the microtome. They are then frozen by CO_2 from a cylinder, or by an ether spray, until of the right consistency for sectioning.

Each of the above methods has advantages for certain kinds of work. The paraffin technique, however, has by far the widest application. Nearly all histological work, normal and morbid, has for its basis the process of inclusion in paraffin,

supplemented, when necessary, by the method of embedding in celloidin, and, still more rarely, by the freezing technique.

2. DEHYDRATION

Before tissues can be embedded by the paraffin or the celloidin methods it is necessary to dehydrate them very thoroughly, since neither of the above substances are miscible with water. To attain this end, up-graded strengths of alcohol are used, the final grade usually being 100 per cent. (*i.e.* absolute) alcohol. The technique varies considerably according to the size, consistency, etc., of the tissues. Generally the objects are passed through up-graded alcohols of 50, 70, 90, and 100 per cent., and remain an equal time in each. As in fixation, the bulk of the fluid (especially of the two last grades) should be much greater than the bulk of the objects.

The various strengths of alcohol should be kept in bottles of 1-litre capacity. Those of 90 per cent. and beneath are best prepared by the dilution of rectified spirit of 96 per cent. and *not* absolute alcohol, the latter being far more costly. Time is saved in preparing the different grades if each bottle be marked with the diamond so as to indicate the levels to which the alcohol and the water need be added to make up the required concentration.

The following table indicates the proportions of 96 per cent. alcohol and water needed to make up the grades commonly employed : [1]

Grade Required.	Amount of 96 per cent. Alcohol.	Amount of Water.
90 per cent.	940 c.c.	60 c.c.
80 „	836 c.c.	164 c.c.
70 „	730 c.c.	270 c.c.
50 „	520 c.c.	480 c.c.

General Procedure of Dehydration.—The tissues are placed in a tube or capsule of the lowest (50 per cent.) grade, *unless*

[1] For the dilution of other grades of alcohol, see the table at the beginning of the book.

they have been fixed in Carnoy's fluid or in some other fixative containing much alcohol, in which case dehydration is begun in 90 per cent. alcohol. The reason why dehydration is usually begun in alcohol of low concentration is that if tissues are taken from either aqueous fixatives or simply water straight into high grade alcohol, serious shrinkage is liable to occur. This, of course, is due to the too rapid exosmosis of water from the cells.

For delicate (i.e. cytological) work, or for large pieces of tissue, dehydrate as follows :

50 per cent. alcohol . .	Twelve to twenty-four hours in each grade. Total time : two to four days.
70 ,, ,, . .	
90 ,, ,, . .	
Absolute alcohol (two baths)	

Or you can put the object into 96 per cent. alcohol between the 90 per cent. and the absolute, thereby obviating the necessity for changing the latter.

For the rapid dehydration of small pieces of tissue—i.e. *not more than several millimetres in thickness—the following times suffice :*

50 per cent. alcohol . .	½ hour	Total time : 2½ hours.
70 ,, ,, . .	½ ,,	
90 ,, ,, . .	½ ,,	
Absolute alcohol (two changes)	1 ,,	

The duration of treatment with the various grades of alcohol has to be modified to suit the circumstances. The above times are only intended to serve as a rough-and-ready guide for the beginner.

Treatment with Absolute Alcohol.—As pointed out by Masson, a better and less wasteful dehydration in absolute alcohol is given by three changes—using only a small volume of alcohol for each change—than by a single bath in a large volume of alcohol.

The reason for this is very simple. Imagine, says Masson, a block of tissue 1 c.c. in volume, placed direct in 99 c.c. of absolute alcohol. The alcohol would be reduced to about 99 per cent. Now, suppose you divided the original 99 c.c. of absolute alcohol into three equal portions, and dehydrated the piece of tissue in each portion in turn. The dilution of the water would pass from 1/33 to 1/33/33, and, finally, to 1/33/33/33, *i.e.* to 0·00003 per cent.

Such vigorous dehydration is never necessary in practice. Three successive baths of absolute alcohol, each of only ten times the volume of the fragment of tissue, will reduce the concentration of water to at least 0·001 per cent. -

In this way, three baths of absolute alcohol, each of 10 c.c., will, in the case of a piece of tissue saturated with water, dehydrate it ten times better, and over three times as economically, as 100 c.c. of alcohol used in a single bath. Furthermore, for the first bath of alcohol you can use alcohol derived from the last two baths—*i.e.* used absolute alcohol.

If you dehydrate in progressive grades of alcohol up to 90 per cent., then two baths of absolute alcohol, each of five to ten times the volume of the tissues, will ensure sufficient dehydration. This method is strongly to be recommended.

Some workers, of whom Masson is one, treat tissues direct with absolute alcohol on taking them out of the fixative. They maintain that no more shrinkage is thereby caused than by progressive dehydration beginning in alcohol of 50 per cent. While this is true for certain tissues, it certainly is not for others. Vesicular structures, and particularly embryological material, are so shrunken by immediate transference from an aqueous fixative to absolute alcohol that the shrinkage is visible to the naked eye.

It must also be remembered that direct treatment with absolute alcohol after an aqueous fixative entails very thorough fixation. Thus, Masson favours fixation in Bouin's fluid for three days as opposed to the twelve or twenty-four hours accorded by most histologists. This entails a loss of time of at least two days, which could perhaps be more profitably spent in progressive dehydration.

3. PRESERVATION OF TISSUES

Tissues should not be kept for longer than the specified time in the fixative. If it be desired to keep tissues for some time before they are embedded, preserve in alcohol of 70 or 80 per cent. Lower strengths macerate tissues, while higher grades are apt to harden them unduly. Note that prolonged preservation of specimens in alcohol is unsatisfactory in that such tissues, when sectioned, stain poorly. Formol-fixed material should be preserved in 3 or 4 per cent. formol, which should be changed at least once. The only

way in which tissues can be permanently kept without deterioration is in paraffin or celloidin blocks.

4. IDENTIFICATION OF SPECIMENS

The bottle or capsule containing the specimens may be labelled in the ordinary manner, but it is safer to place a small pencilled label *inside* the vessel containing the tissues. When the latter are moved from one capsule, etc., to another, the label accompanies them, and, when they are finally embedded, the label is fixed on to one side of the paraffin mass. A stock of such labels—easily prepared by cutting paper into small oblong pieces—should be kept. The use of this method is the only sure means of obviating confusion when many different pieces of tissue are being treated simultaneously.

It is equally necessary to keep some record of the solutions through which objects are being passed. This may be done either by placing the tube or capsule containing the tissues on a piece of paper bearing the necessary indications, or, more simply and surely, by writing on the vessel with a grease pencil.

5. TECHNIQUE OF PARAFFIN EMBEDDING

Paraffin wax being nearly insoluble in alcohol, it is necessary to replace the latter by some substance in which paraffin is soluble, before proceeding with the actual impregnation of the tissues.

Embedding in paraffin thus comprises two processes : (*a*) the process of *clearing—i.e.* the substitution of a paraffin solvent for the alcohol, and (*b*) the *impregnation* of the object with paraffin wax.

The term " Clearing " has arisen from the fact that the reagents used for the purpose of eliminating the alcohol have the effect of increasing the refractive index of tissues, thereby making them relatively transparent.

A. CLEARING

The substances most commonly employed are xylol, toluol, chloroform, and oil of cedar. The *modus operandi* is the same with all : the absolute alcohol is poured away

and a relatively large volume of the clearing reagent is added to the tube or capsule containing the objects. For large objects the clearing medium should be changed at least once. The time required for clearing varies according to the size and nature of the pieces of tissue and the particular clearing reagent employed.

Xylol, for example, clears as follows :

Small objects (*i.e.* not thicker than 5 mm.).	Half an hour to one hour.
Larger objects (up to 1 cm. in thickness) .	Three to six hours.
Very bulky specimens (whole embryos, etc.)	Twelve hours.

Keep tissues in xylol for the minimum time—*i.e.* only as long as is necessary to remove the alcohol—since prolonged treatment (*e.g.* several days) with this reagent makes objects brittle.

Xylol is relatively rapid in its displacement of alcohol. *Toluol*, *Chloroform*, and *Oil of Cedar* are slower in their action as clearing media.

Toluol is useful in that tissues can be left in it for twelve to twenty-four hours without fear of their becoming brittle.

Chloroform takes two to three times as long as xylol to clear, but its hardening effect is less.

Cedar Oil is admirable in that tissues are not hardened even by prolonged immersion in it. It clears from 95 per cent. alcohol, but has the disadvantage—only noticeable with large objects—of slow penetration and subsequent difficulty of elimination in the paraffin bath. Even small pieces of tissue should be left overnight in this medium.

To clear very delicate tissues (*i.e.* thin-walled cysts, cytological material, etc.) use one of the following methods : (*a*) add xylol, or other clearing medium, in small amounts to a little absolute alcohol containing the tissues. Continue until the mixture is nearly pure clearing medium. Then transfer to the latter. Usually it suffices to place the objects in a mixture of equal parts of clearing medium and absolute alcohol before transferring them to the pure clearing medium. (*b*) Half fill a tube with cedarwood oil. Then carefully add on top of the oil an equal volume of absolute alcohol with a pipette, avoiding mixture of the oil and the alcohol. Then

add the specimen, which floats at the interface of the two liquids, becomes slowly permeated with the oil, and finally sinks to the bottom, when clearing is complete. Finally transfer the tissues for a short time to pure oil of cedar—unless they are very small.

B. INCLUSION IN PARAFFIN

The following are needed : (i) *An Embedding Oven or Thermostat.* The usual pattern consists of a water-jacketed copper cupboard provided with some means of keeping constant the temperature inside it. In the older embedding baths this is effected by a mercury regulator, but more recent models of the Hearson pattern have a capsule regulator similar to that employed in biological incubators. In all cases the controlling mechanism is adjusted so as to keep the temperature inside the thermostat slightly above the melting-point of the wax. It is important never to use the embedding bath to warm watery solutions (unless well corked), as the presence of water vapour inside it destroys the chances of successful embedding. The temperature inside the thermostat should be from 55° to 66° C. (*i.e.* 131° to 140° F.). A temperature of 58° to 60° C. suffices for most purposes.

(ii) *Paraffin Wax* of a suitable melting-point. Paraffins of high melting-point are hard when allowed to cool, those of a low are soft. The choice of a paraffin of the right degree of hardness depends upon two factors :

(*a*) *The Nature of the Object*—i.e. *whether it be hard or soft.* For *hard objects* it is necessary to use a hard paraffin in order to support the tissues sufficiently during section cutting. For *soft objects* a relatively soft paraffin is employed.

(*b*) *The Temperature at which the Tissues are Sectioned.*—The degree of hardness of the paraffin block containing the object is obviously dependent on the surrounding temperature. Thus, in summer or in tropical climates it is necessary to use a paraffin of a high melting-point in order that it should be of the right consistency for sectioning.

In practice the above works out as follows : For ordinary work in temperate climates a paraffin with a melting-point of 50° C. (122° F.) is satisfactory. For hard objects or for

section cutting in very warm weather, wax of 55° C. (131° F.) is necessary. Finally, for work in the Tropics, paraffin of a melting-point of 60° C. (140° F.) is indicated.

It is important that the paraffin used for embedding should be clean; the blocks as purchased are not usually so. To remove dust, grit, and other extraneous matter, filter the molten wax through an ordinary filter paper in the thermostat. Keep the paraffin in enamelled jugs inside the thermostat. For work in temperate climates it suffices to keep only two grades of paraffin in stock—45° C. (113° F.) and 55° C. (131° F.) M.P. For ordinary purposes use a mixture of equal parts of these, while for embedding hard material, or for work in very warm weather, employ the paraffin of 55° C. M.P. alone.

Procedure with Paraffin.—The object is assumed to have been cleared (as described on p. 48); it remains to remove the clearing reagent and to permeate the tissues with the paraffin. This is done in one of two ways.

(*a*) *For delicate work* transfer the object to a mixture of the clearing medium and paraffin—*e.g.* xylol-paraffin, cedar oil-paraffin, etc. Such a mixture is prepared by adding paraffin shavings to the clearing medium in a tube or watch-glass. Transfer the tissues to this and place in the thermostat. As the clearing medium warms up, it dissolves the paraffin, and the objects become permeated with the mixture. Do not throw away the clearing medium + paraffin, as it can be used over and over again.

(*b*) *For rapid work with ordinary material* the intermediate treatment outlined above may be omitted. The tissues are transferred straight from the clearing reagent to molten paraffin wax in a glass tube or a very small copper saucepan.

The Paraffin Bath.—This involves soaking the tissues in pure molten paraffin, which should be changed at least once.

The duration of the paraffin bath is dependent (*a*) on the size—and especially the thickness—of the object, and (*b*) on the texture of the tissues—*i.e.* whether loose or dense. Obviously it takes a longer time for the paraffin to penetrate right through large and dense objects than through thin ones of a reticular texture.

The following times are only intended as an approximate indication for the beginner :

Thickness of Object.	Time in Clearing Medium + Paraffin (optional).	Total Time in Pure Paraffin.	Number of Times the Paraffin should be changed.
Under 3 mm. ..	¼ to ½ hour.	½ to 1 hour.	Once.
„ 5 mm. .	¾ to 1 „	1 to 2 hours.	Once or twice.
„ 1 cm. .	1 to 2 hours.	3 to 6 „	At least twice.
Very bulky objects — *e.g.* whole embryos, etc.	Several hours to overnight.	Leave overnight, *e.g.* 12 or more hours.	At least three times.

For urgent work, fix and embed by the method outlined on p. 317.

The golden rule in all successful embedding is to keep the objects in the thermostat for just as long a time as is necessary—and neither more nor less. If any of the clearing medium is retained in the tissues, or if the paraffin has failed to penetrate them—these being the two effects of insufficient duration of the paraffin bath—the tissues will not section properly. On the other hand, prolonged exposure to the heat in the thermostat causes shrinkage and hardening of tissues—whence difficulty in section cutting.

The following tissues, on account of their compact nature, need relatively lengthy treatment in the paraffin :

Brain and spinal cord } Impregnate overnight in paraffin for medium-
Skin . . . } sized pieces (5 to 10 mm. thickness).

Tissues for which it is important to reduce the duration of the paraffin bath to a minimum, because of its hardening effect on them, comprise :

Muscle—striated, non-striated, and cardiac .
Fibrous tissue . . . . . .
Organs containing much blood—*e.g.* spleen .
Pathological material containing thrombi, emboli, etc. (Fibrin becomes very hard during dehydration, clearing, and embedding.)

} Impregnate in paraffin for one hour (maximum). Vacuum embedding is indicated for such material. Whenever possible take very thin pieces of tissue.

It is usually essential to remove all traces of the clearing reagent from the object by a sufficient number of changes of the paraffin, otherwise section cutting suffers. Note, however, that a trace of cedar oil in the paraffin block *aids* sectioning, in that it imparts a slightly greasy quality to the wax which is often advantageous.

Casting.—By this process the object is first placed in a mould of suitable dimensions filled with paraffin ; next it is orientated so as to ensure its being sectioned in the right plane ; and finally the paraffin containing the tissues is cooled, thereby causing it to "set." When all this has been done, the object is ready to be sectioned.

Types of Mould employed.—For very small objects *watch-glasses* are useful, while larger pieces of tissue may be cast in *round glass capsules.* The inner surface of the vessel is *thinly* smeared with glycerine with the finger tip. This ensures that the block does not adhere to the glass.

Leuckhart's Embedding-Boxes are very convenient. A set composed of several sizes of these answers the requirements of any laboratory. By sliding the L-shaped pieces of metal the one against the other on a glass plate, the shape of the block can be varied in accordance with the dimensions of the object.

Paper Boxes can also be used. The only disadvantage of this method is the time required to make the box.

For most work Leuckhart's embedding-boxes are the most useful, supplemented, for very minute objects, by the watch-glass method.

Orientation of the Object.—This is done with the aid of a forceps, the tip of which is gently warmed in the Bunsen burner. Unless this precaution be taken the wax immediately solidifies around the forceps, the object clings to it, and deft manipulation is impossible. Do not heat the tip of the forceps until red hot, or the tissues will be charred.

PROCEDURE.—*Note.—The following manipulations must be performed quickly, since the paraffin in the mould solidifies very rapidly.*

1. Fill the mould (watch-glass, embedding-box, etc.) with molten paraffin.

2. Place the object or objects in the bottom of the mould

with the warmed forceps. Then orientate them so that they are conveniently situated for trimming the paraffin block prior to sectioning. This is best done by placing the object so that the part of it at which sectioning is to be begun lies face downwards (see Fig. 3). For trimming, invert the block ; the piece of tissue will then be near the surface, while the greatest thickness of paraffin will be beneath it, which is an advantage, as this side has to be melted in order to fix the block on to the object-holder. Several different objects may be embedded in the same mould in different parts of it.

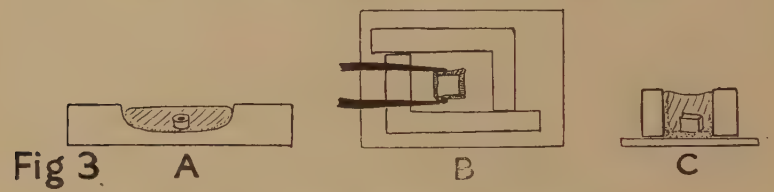


Fig. 3.—Casting Tissues in Paraffin.

A shows the watch-glass method in vertical section. The paraffin rapidly forms a solid layer at its point of contact with the glass (dotted). The object is then orientated with a warmed forceps on this layer. If the concavity of the watch-glass has been thinly smeared with glycerine, the paraffin will usually detach itself when cooled in water. A glass capsule may be used for larger objects in the same manner.

B. Casting the object in a Leuckhart embedding-box composed of two L-shaped pieces of brass resting on a glass plate. Orientation of the object is effected with a warm forceps. As seen from above.

C. The same in vertical section. The object has been orientated upon the layer of semi-solid paraffin formed at the surface of the glass plate.

But enough space must be left around each object to enable it to be cut out completely enclosed in a piece of paraffin with parallel sides, if you wish to section each piece of tissue separately.

3. Fix the label, which should have accompanied the tissue into the paraffin bath, on to one side of the block by pressing an edge of the paper against the solidifying wax in a corner of the mould.

4. Blow gently on the surface of the paraffin, or put aside the mould to cool, until a thin film of solid wax is formed on the surface.

5. Gently immerse the mould in a beaker or sink of cold water to cool the paraffin rapidly. This saves time and also prevents crystallisation of the paraffin, which is a bar to successful section cutting.

When placing the mould in the water, always tilt it slightly, so that it is progressively covered by the water from one end to the other. The deep pits often seen on the top of paraffin blocks are due to the neglect of this precaution ; consequently the thin surface layer of semi-solid paraffin is ruptured by the too sudden contact with the cold water.

6. When the block is solid, remove it from the water. Specimens cast in glycerinised watch-glasses or capsules usually float out of their own accord owing to the contraction of the paraffin when cooling. But if necessary free with a scalpel or spatula. Objects cast in embedding-boxes can be detached by throwing the block plus the metal moulds (after the glass plate has been detached) on to a bench or stone slab. The objects are now ready for sectioning. Tissues may be kept indefinitely once they have been " blocked." It is, in fact, quite the best way of preserving material.

Remember that the paraffin must have set completely before you attempt to trim it. Paraffin is a very bad conductor of heat. Therefore, even a small block needs to be left in the water for ten to fifteen minutes in order to ensure solidification of its centre. Large blocks require half an hour to one hour. Provided the tissue is properly embedded no harm is done if the paraffin mass be left overnight in the water.

6. VACUUM EMBEDDING

Vacuum embedding, or, more accurately, embedding under reduced pressure, is of use for certain purposes. A special type of thermostat is necessary in which the embedding chamber is so arranged as to be airtight. The procedure consists in partially reducing the pressure inside the thermostat during the process of impregnation with the paraffin. The necessary reduction in the pressure is effected either by a hand-worked exhausting pump or by means of a water aspirator.

The advantages of the method are as follows:

1. The time required for embedding is considerably reduced, the clearing reagent being more rapidly replaced by the paraffin than with the ordinary method of inclusion. This reduction in the time is a great advantage in the case of those tissues which become unduly hard during the process of embedding.

2. Any air present in the tissues is extracted.

Apparatus Required.—In the Hearson vacuum thermostat the embedding chamber is a circular brass well, closed at its upper end by a thick glass plate lying upon a rubber ring. This chamber is kept at the proper temperature by a water jacket, while a small tube places it in communication with the exhausting apparatus (vacuum pump or aspirator). There is also an arrangement, in the shape of a small screw plug, fitted into the wall of the chamber, whereby air can be admitted to it. When the apparatus is used in conjunction with a water aspirator, a glass bottle or a thick-walled flask of at least 500 c.c. capacity should be interposed between aspirator and thermostat. This serves to avoid water being sucked directly into the embedding chamber should the water supply to the aspirator be inadvertently turned off before opening the air valve. Finally, it is advantageous to attach to the apparatus some form of gauge—*e.g.* a mercury manometer. Fig. 4 shows the necessary installation.

Procedure.—1. Place the tissue in pure paraffin within the thermostat.

2. Reduce the pressure *very slowly*. A reduction to 400 or 500 mm. Hg usually suffices. The time required for impregnation varies from one-quarter to one-half of that required for the same objects at atmospheric pressure.

3. *Slowly* admit air through the air valve, until the pressure within the thermostat is in equilibrium with that of the atmosphere. For large objects, repeat the process of exhaustion two or three times, changing the paraffin in between.

To ensure success the exhaustion must be very gradual, so likewise the readmission of the air. Delicate air-containing tissues are ruined by a too rapid exhaustion. The effect of rapidly exhausting lung, for instance, is to burst the alveoli,

thereby causing an artefact bearing the closest resemblance to genuine pulmonary emphysema.

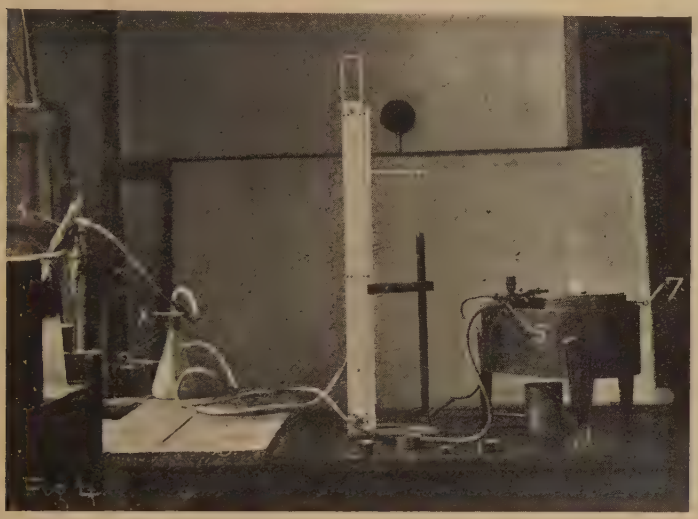


FIG. 4.—ARRANGEMENT OF A VACUUM EMBEDDING-BATH.

1. Water aspirator connected to:

2. A thick-walled flask. This prevents water, in the event of a diminution of the water pressure, being sucked back into the thermostat.

3. A T-piece connected with the mercury manometer and the thermostat.

4. The manometer. A long U-tube, one end of which is inverted in a tube of mercury.

5. Tap cutting off the interior of the thermostat from the exhausting system.

6. Glass plate over the vacuum chamber of the thermostat.

7. Screw plug serving to admit air to 6.

On the table: copper pans used for the impregnation of tissues in the thermostat.

To exhaust: turn on 1; open 5; close 7, and press on 6 until air pressure begins to diminish.

To admit air: turn off 5; open 7 to admit air slowly. Disconnect X and turn off the tap 1.

N.B.—Avoid turning off tap 1 while there is free communication between it and the thermostat, or water will be sucked in.

The vacuum method is indicated in the following instances:

1. For air-containing tissues, and especially lung, for which it is often a necessity.

2. For large or dense objects, *e.g.* whole embryos or large pieces of brain.

3. For tissues which become unduly hard if the embedding bath be at all prolonged, *e.g.* fibrous tissue and muscle.

4. For any work where speed is of importance.

7. TABLE OF FAULTS IN DEHYDRATION, CLEARING, AND EMBEDDING

Fault.	Diagnosis.	Remedy.
Insufficient dehydration.	Clearing reagent turns milky as soon as tissues are placed in it.	Repeat dehydration with absolute alcohol. Then clear again.
Insufficient clearing.	Tissues are opaque. Section cutting difficult or impossible, owing to presence of alcohol.	Repeat the clearing process. If object has already been "blocked," prolonged clearing (*e.g.* twelve hours or more) is necessary. Then re-embed.
Insufficient impregnation with the paraffin.	Paraffin block (after cooling) is moist and crumbles. It also smells of the clearing reagent. Sectioning difficult or impossible.	Repeat the process of impregnation with the paraffin. Change latter at least once. Then cast.

8. SCHEME OF THE PROCESSES INVOLVED IN PREPARING TISSUES FOR SECTIONING BY THE PARAFFIN METHOD

Fixation . . . (Chapter IV.)

↓

Washing in water . Only necessary after fixatives containing OsO_4, chromic acid, or chrome salts.

↓

Dehydration in alcohol of . .
- (1) 50 per cent. (2) 70 per cent. — If fixative contains $HgCl_2$, add iodine to these.
- (3) 90 per cent. — If fixative be strongly alcoholic, *e.g.* Carnoy, start dehydration in this grade.
- (4) Absolute. — Change at least once.

↓

Clearing . . Xylol, toluol, or cedar oil.

↓

Inclusion in paraffin by ordinary or vacuum methods . .
- (1) Permeation with mixture of clearing medium + paraffin. (*Optional.*)
- (2) Permeation with pure paraffin. (Change at least once.)
- (3) Casting the tissues. (Orientation and cooling the paraffin mass.)

↓

Sectioning . . (Chapter VI.)

9. CELLOIDIN EMBEDDING

Celloidin is a purified form of collodion or nitro-cellulose. Its main advantage over ordinary collodion is that it is more transparent. The tissues are placed in ether-alcohol solutions of celloidin of increasing strengths after careful dehydration. Clearing is usually omitted. Once the object is thoroughly permeated with the celloidin, the mass is hardened by exposure to chloroform until of the right consistency for section cutting. All celloidin embedding is done at room temperature.

Utility of the Method.—Inclusion in celloidin is indicated in the following instances :

1. *For Very Large Objects.*—These, when treated by the paraffin method, are apt to become very hard, since prolonged immersion in the paraffin is necessary to ensure thorough penetration. Also, when the surface to be sectioned is large, paraffin sections are liable to break or roll during the process of cutting.

2. *For the Inclusion of Hard Tissues* (*e.g.* pieces of decalcified bone, etc.).—Celloidin supports such tissues (on account of its gutta-percha-like consistency) far better than paraffin, which tends to crumble.

3. *For Embedding Pieces of Central Nervous System*, when using special silver and other impregnation methods. With certain of these methods it is sometimes necessary to avoid heat. For such work inclusion in celloidin is the standard technique.

4. *For Preserving the Relations of Cell Layers of Different Consistency* (*e.g.* the Eye).—In sections of this organ made by the paraffin method, the retina always becomes detached from the harder tissues (choroid and sclerotic) encircling it.

The Disadvantages of Celloidin (as compared with paraffin) are as follows :

1. It is not possible to cut very thin sections. 10 μ is the minimal thickness for most material, while 15 to 20 μ is the usual thickness.

2. The process of inclusion in celloidin is very slow—a matter of days or weeks.

3. The preparation of serial sections is difficult and demands great skill. This is because each section has to be cut and stained separately—except in such cases in which the celloidin-paraffin method (see p. 62) can be used.

Preparation of the Celloidin Solutions

Celloidin is supplied in shreds which are usually preserved in water. Before use, dry the shreds thoroughly on blotting-paper on top of the thermostat.

For ordinary purposes two celloidin solutions suffice. Prepare these thus (as advised by Mallory and Wright) :

Dissolve 30 grammes of dry celloidin in 200 c.c. of a mixture of equal parts of ether and of absolute alcohol. This will give you the thick solution. Dilute 50 c.c. of this with 50 c.c. of the ether alcohol. This will constitute the thin solution.

NOTE.—Celloidin dissolves more rapidly if it be first placed in the absolute alcohol for several hours before adding the ether. Keep the two solutions in well-stoppered bottles, and remember that celloidin mixtures are highly inflammable.

A. THE "WET" CELLOIDIN PROCESS

Work, if possible, with pieces of tissue not thicker than 5 mm., preferably from 2 to 3 mm. Embedding is thereby accelerated. Use a well-stoppered bottle or tube, as even a trace of water vapour will inhibit penetration.

After thorough dehydration in absolute alcohol proceed as follows :

1. *Soak the object* for twenty-four hours in a mixture of equal parts of *Ether and Absolute Alcohol.* The aim of this is to ensure easy penetration by the celloidin.

2. *Impregnate with the Thin Celloidin Solution.*—This requires at least twenty-four hours, usually several days, and sometimes several weeks. The time is dependent on the thickness and consistency of the tissues.

3. *Impregnate with the Thick Celloidin Solution* for an equal time.

4. *Harden the Celloidin Mass.*—This should be done in a desiccator and the object taken out of the tube or bottle and placed either in a paper box or in a mould made by pinning a paper band round a cork of suitable dimensions. Hardening is effected in two stages.

(i) Lift the cover of the desiccator every day for a few seconds to assist the thickening of the celloidin. If the object is left dry during this process, add more of the thick celloidin solution to cover it. As soon as the mass " has attained a consistency such that the ball of the finger (*not* the nail) no longer leaves an impression on it " (Bolles Lee), remove the paper and proceed with stage (ii) of the hardening process as recommended by Bolles Lee.

(ii) Add a few drops of chloroform to the bottom of the desiccator. The effect of the chloroform vapour is to harden the celloidin. This process requires a few hours (for small objects) to several days (for large ones). Once the superficial layers of the celloidin have set, even and rapid hardening is facilitated by taking the celloidin mass out of the mould, and exposing it to the chloroform vapour.

5. *Preserving the Blocks.*—Keep the blocks in 80 per cent. alcohol until required for sectioning. It is simplest to mount

them (after trimming away the superfluous celloidin) on vulcanite blocks before preserving in alcohol. To do this, dip the base of the celloidin block in thick celloidin, press it firmly on to the vulcanite block, and treat with chloroform or chloroform vapour for half an hour.

B. THE "DRY" CELLOIDIN METHOD

The technique just described, although the standard one, has the disadvantage that the sections have to be cut " wet " —that is to say, both knife and block have to be kept flooded with alcohol during the sectioning process. It is also necessary to preserve the blocks in 80 per cent. alcohol. With the following technique both cutting and storing in alcohol are obviated. But many workers prefer the " wet " process—especially for large sections.

PROCEDURE.—Take the tissues through stages 1 to 4 as described above. When the celloidin is *nearly* of the right consistency (but not quite, as the following process hardens somewhat) place the mass in *Gilson's mixture*. This is composed of equal parts of chloroform and cedar oil. Add oil at intervals of one hour (for small objects) to one day (for large ones). Continue doing so until the mixture is about 90 per cent. of oil of cedar. By this time the celloidin should be quite transparent. Then expose to the air until dry. Fix the block on to the vulcanite holder as already described, but expose to chloroform *vapour* for only a short time (a few minutes).

The block can now be cut dry, a great advantage over the wet technique. Blocks are preserved by keeping them in a dry stoppered bottle.

10. DOUBLE-EMBEDDING

It requires considerable skill and much time to obtain serial sections of material embedded in celloidin. The following technique enables serial sections to be cut in ribbons, and then stained on the slide. The paraffin-celloidin method gives the best results with small objects.

PROCEDURE.—(1) Embed in celloidin by the "dry" method. Trim the celloidin block so that it forms a rectangle just enclosing the object.

(2) Transfer the celloidin mass from the cedar oil into paraffin. Leave in the thermostat for one hour.

(3) Orientate and cast the celloidin mass (containing the object) in the usual way.

CHAPTER VI

SECTION CUTTING

A. Introductory (p. 64).
B. Mechanical Equipment (pp. 65 to 74): 1. Types of Microtome —2. The Knife—3. Stropping and Honing—4. The Relation between Knife and Object (Slant and Tilt).
C. Paraffin Sections (pp. 74 to 84): 1. Preparation of the Embedding Mass—2. Preparation of the Microtome—3. Cutting the Sections—4. Manipulation of the Ribbon—5. Fixing Sections on the Slide—6. Table of Errors in Section Cutting and their Remedy—7. Additional Notes.
D. Celloidin Sections (pp. 84 to 85): 1. Sectioning by the "Wet" Method—2. Sectioning by the "Dry" Method—3. Fixing Celloidin Sections on the Slide.
E. Paraffin-Celloidin Sections (Double Embedding) (p. 86).
F. Frozen Sections (p. 86): 1. Preparatory Treatment—2. Sectioning.

A. INTRODUCTORY

There are few aspects of histological technique in which personal experience plays so large a part as in the cutting and manipulation of sections.

It is necessary to become familiar with all the adjustments of your microtome before attempting to cut sections with it. Then try to make serial sections of some tissue which cuts fairly easily. Small pieces of fœtal material, which contains relatively little fibrous tissue, are suitable for such practice.

Remember that a microtome is a delicate and usually complicated piece of mechanism. Like a motor-car, it is only when the manipulation of the microtome has become a series of "reflex actions," and when you are familiar with all its idiosyncrasies, that real competence has been attained.

Patience, common sense, and a good knife edge form the the basis of all good section cutting.

B. MECHANICAL EQUIPMENT

1. **Types of Microtome.**—There are many types of microtome. Three of the most common types are illustrated here.

FIG. 5.—CAMBRIDGE ROCKING MICROTOME (LARGE MODEL) AND THE EQUIPMENT REQUIRED FOR CUTTING PARAFFIN SECTIONS.

1. Knife-holder.

2. Chuck clamped on to a ⊃-shaped arm. This arm moves up and down in the arc of a circle in front of the knife edge.

3. Pulley wheel on to which is clamped a length of catgut; the latter is connected to lever 7. By pulling the latter backwards and forwards the object is moved up and down in front of the knife edge.

4. Arm bearing on a sleeve attached to a long micrometer screw (10), on the base of which is mounted a ratchet wheel (9). Every time handle 7 is pulled, pawl 5 engages with 9, the sleeve on 10 is very slightly lowered, and arm 4 with it. This causes the object arm (3) to be very slightly advanced. 6 is another pawl also engaging in ratchet wheel 9; it retreats the object very slightly at the beginning of its passage up the knife. The idea of this is to avoid the object and the paraffin (or celloidin) block rubbing against the knife on the upward stroke.

8 is the adjustment for thickness of the sections.

Shown in the foreground of this figure are: the capsule of glycerine and albumin solution, the scalpel, brush, and forceps for manipulating paraffin sections, and an instrument for melting the under-surface of the paraffin block when sticking it on the object-holder. A ball-and-socket object-holder, enabling the object to be orientated, is also shown.

(1) *The Cambridge Rocker.*—Of this there are two types in common use: the large pattern, illustrated in Fig. 5, and a smaller model used in most laboratories in Great Britain.

The latter cuts not flat but slightly curved sections, and is inferior to the large model in performance. Both instruments lack any means of rationally adjusting the knife, and, furthermore, will not accommodate a large one. To obtain good results with the Cambridge rockers, the movement of the

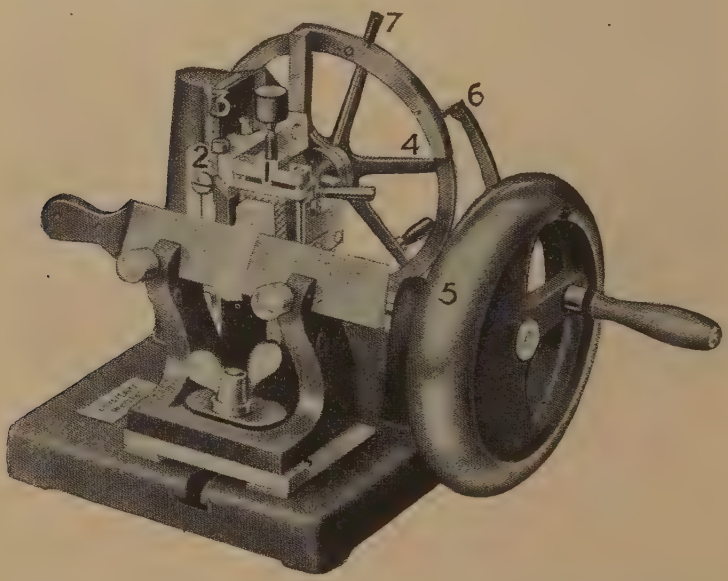


FIG. 6.—ROTARY MICROTOME OF LEITZ, WITH KNIFE IN POSITION FOR CUTTING PARAFFIN SECTIONS.

1. The object-holder.
2. The object-slide moving vertically up and down pillar 3.
4. The ratchet wheel, which is slightly rotated, thereby advancing the object, every revolution of flywheel 5 through pawl 6.
7. The adjustment of the thickness of the sections (1 to 20 μ).

The knife is adjusted for cutting paraffin sections in this figure.

object against the knife needs to be rather abrupt. The small instrument will only cut paraffin material, but the large model can be usefully adapted, by means of a special knife-holder, to cut celloidin sections also.

(2) *The Rotary Microtome.*—The most suitable microtome for general work. Every laboratory should possess one.

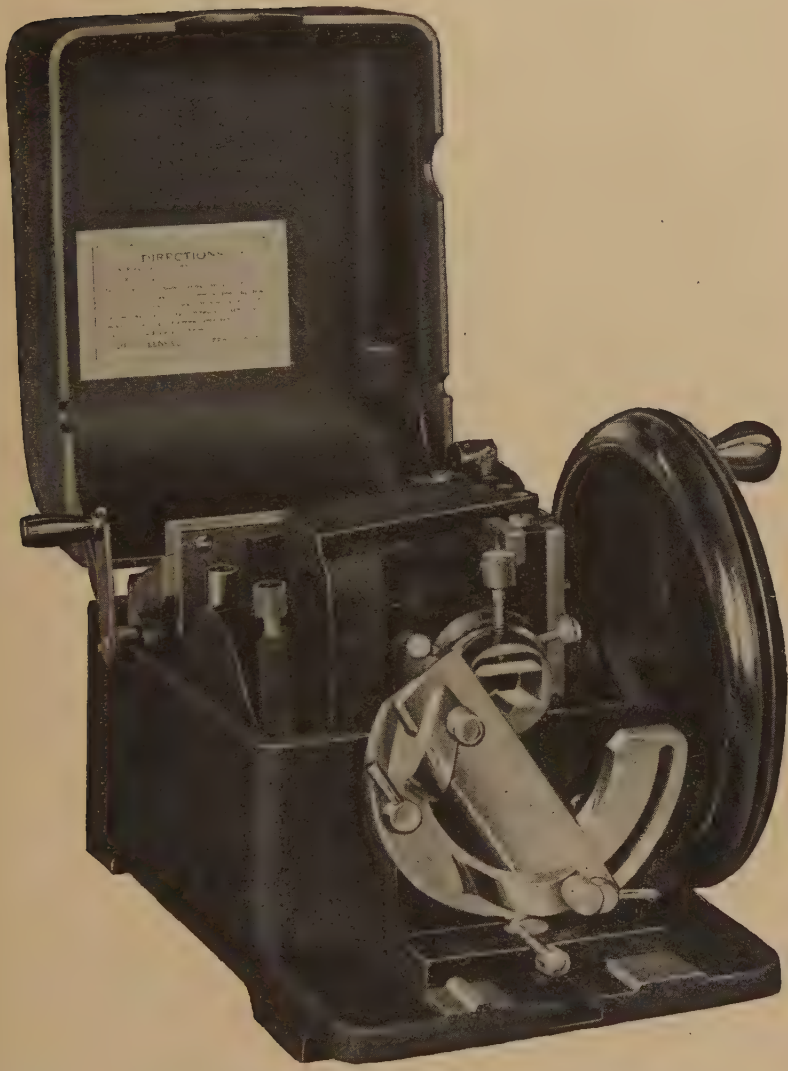


FIG. 7.—LARGE MODEL SPENCER ROTARY MICROTOME, WITH OBLIQUE KNIFE FOR CUTTING CELLOIDIN SECTIONS.

As in the preceding machine, the object moves vertically up and down. A slanting knife, as used for cutting celloidin sections, is illustrated. Orientation of the object is secured by a ball-and-socket adjuster. Thickness of sections 1 to 50 μ.

The first instrument of this type was designed by the late Professor Minot; hence the name "Minot microtome" often applied to these machines. Examples are shown in

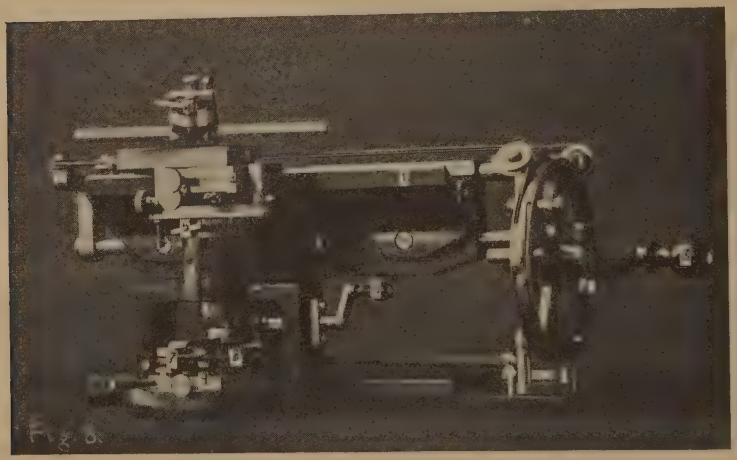


FIG. 8.—JUNG SLIDING MICROTOME.

In this instrument the object is fixed and the knife movable.

1. Knife slide.

2. Adjustable knife clamp with a planoconcave knife in position for cutting celloidin sections. Beneath the knife-holder is the knife carriage, connected by a U-shaped loop of stout catgut to handle 3. A to-and-fro movement of the latter causes the knife to slide backwards and forwards along the slide.

4. The adjustable object-holder; the wooden cube in this is one such as is used for the attachment of paraffin or celloidin blocks.

5. Clamp for sleeve, which enables the whole object-holder to be raised or lowered.

6. Fine adjustment for regulating height of object-holder.

7. Pin and elbow clamp fitting over it; when disconnected the feed mechanism of the micrometer screw is thrown out of action.

8. The ratchet wheel engaged by a pawl. By means of the micrometer screw beneath 8 the object-holder is raised every stroke of the knife.

9. Adjustment of thickness of sections (1 to 60 μ).

Figs. 6 and 7. Though primarily designed for paraffin, instruments of this type can be used for cutting celloidin, provided that the knife be fixed diagonally in a special holder. A steady motion of the flywheel is needed to obtain good ribbons.

(3) *The Sliding Microtome* (see Fig. 8).—Best reserved

for special purposes, such as celloidin work and the cutting of refractory paraffin material. In the majority of these instruments the knife moves against the object, which is fixed. This is in contrast with types 1 and 2. A slow but very steady motion of the knife is needed with sliding microtomes. More experience is needed to manipulate this instrument than the rotary machine or the Cambridge rocker.

(4) *The Freezing Microtome.*—This instrument is used for cutting unembedded tissues which have been frozen to the right consistency with carbon dioxide. Fig. 12A illustrates this model, which is described on p. 88.

2. **The Knife.** — A sharp knife is the most important requirement in all section cutting. Jagged edges on the knife mean tears or striations in the sectioned tissues. It must be remembered that defects such as these, however insignificant they may appear to the naked eye, are intensified many hundreds of times on the tissues when these are finally examined with the high power of the microscope. A really good knife edge should not only feel sharp when tested against the skin of the hand in the usual way, but when examined with a low ($\times 50$) power of the microscope should show no teeth.

The best knives for section cutting are those with a broad base (so as to reduce vibration to a minimum) and a length of at least 15 cm. (6 inches). The longer the knife the better, for, after one portion of the edge has become blunt, it is only necessary to slide the knife along the knife-holder to bring a sharper part of the edge into action. In this way stropping and honing are reduced to a minimum.

The chief types of microtome knife are as follows :

(1) *The Plane-Concave (Hollow-ground) Type.*—Used only for celloidin work.

(2) *The Plane-Concave (less Concave than No.* 1).—Used for paraffin work with the sliding microtome. Also applicable to paraffin material with rotary microtomes, though model 4 is preferable with these machines.

(3) *The Plane-Wedge Knife.*—Restricted to frozen sections.

(4) *The Biconcave (Minot) Type.*—Used for paraffin work with rotary microtomes.

See Fig. 9 for the profiles of these knives.

Care of the Knife.—After use, clean off the paraffin with xylol. Dry by lightly pressing between two folds of a duster, drawing the edge away. It is better to keep two knives—one for rough, the other for fine work.

3. **Stropping and Honing.** — When a knife has become blunt, but is devoid of teeth, it is usually only necessary to strop it. For delicate work the knife is stropped before every object is sectioned. After prolonged use the edge becomes

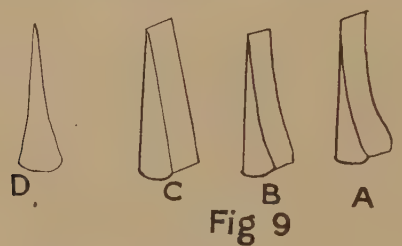


FIG. 9.—TYPES OF MICROTOME KNIFE SEEN IN PROFILE.

A. Plane-concave (*hollow ground*). *Uses*: Celloidin material only.

B. Plane-concave; here the concavity is less than in A. *Uses*: Paraffin sections with sliding microtomes. It can also be used for paraffin material on rotary machines, though type D is preferable for this.

C. Plane-wedge type. *Use*: Frozen sections only.

D. *Minot pattern*; slightly biconcave. *Use*: Paraffin sections with rotary microtomes.

NOTE.—The surface of the knife which bears against the tissues is, in all these figures, on the left.

damaged to such an extent that stropping alone is of no avail, and the knife has to be honed.

And, finally, it is occasionally necessary to regrind the knife. This can only be done by a competent technician, and is beyond the capacity of the laboratory worker. Stropping and honing, however, are within the reach of most people after sufficient practice. These processes may be briefly outlined as follows :

When *stropping* a knife, always draw it *away* from the edge—i.e. *back foremost*. At the end of every stroke turn the knife over so that each side is stropped in turn. If—as is

usually the case—the knife is longer than the strop is broad, move it tangentially over the surface of the strop during every stroke as indicated in Fig. 10, B. If the edge is only slightly dulled, some half-dozen strokes on the harsh, and another dozen on the smooth, surfaces of the strop will suffice. Never

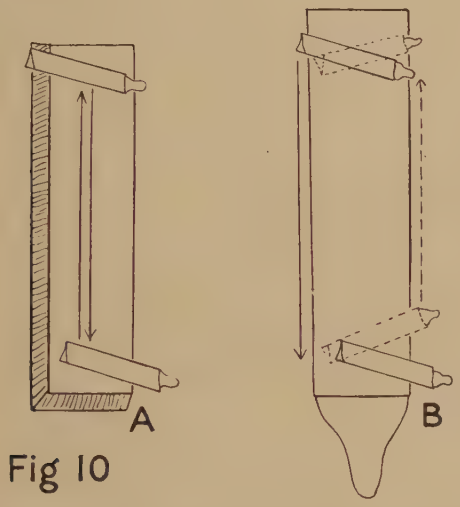


FIG. 10.—HONING AND STROPPING MICROTOME KNIVES.

A. *Honing.*—The knife is gently and evenly pressed, *edge first*, from one end of the hone to the other. In the case of Minot or plane-wedge knives, the knife is turned over so as to sharpen the other surface every ten to fifty strokes. For plane-concave knives only the concave surface should be rubbed on the hone. Since the knife is often wider than the hone, a lateral movement must be made during each stroke so as evenly to sharpen the entire knife edge.

B. *Stropping.*—The movement is the reverse of honing—*i.e. edge last.* In the case of wedge, plane, and Minot knives, the knife is turned round at the end of each stroke so as to sharpen each surface alternately. For plane-concave knives only the concave surface should be honed. Stropping requires a good deal of practice.

press hard against the strop with the knife, or you will blunt the knife instead of sharpening it. Usually the weight of the knife itself suffices.

When *honing*, hold the knife by both ends on a hone. The surface of the latter is covered either with oil or soapy water. Liquid paraffin (B.P.) is also excellent.

Place the knife at one end of the hone. Then push it *edge first* (in contrast with stropping) to the other end. Next, turn the knife over and bring it, on its other surface, to the extremity of the hone. Hold the knife obliquely and take the same precautions to hone the entire length of the knife as when stropping it. The necessary movements are indicated in Fig. 10, A. Honing is continued until all the teeth on the knife edge have been eradicated. This may take half an hour or more. Never press the knife hard on to the stone. If light pressure will not obliterate the teeth, use a harsher stone.

After honing, wipe off the oil or soap from the knife with xylol. Then strop it thoroughly. The hone should be kept protected from dust when not in use.

Note that when honing or stropping knives other than those of the biconcave (*i.e.* Minot) variety, it is necessary to place a special " bevel " or back on to the base of the knife. This is a semicircular steel sheath fitting over the base of the knife. Its aim is to hold the knife edge at such an angle as will enable it to acquire a good cutting surface.

The aim of stropping is to polish and sharpen the knife edge, that of honing is to remove irregularities from the knife edge.

4. **The Relation between Knife and Object.**—This is very important. The relation is twofold, since it involves the relation of knife to object in both horizontal and vertical planes.

By *slant* is meant the angle along which the knife edge attacks the paraffin or celloidin block. In practice the knife is used either square or slanting.

(A) Slant.—A square knife means one the edge of which is parallel to upper and lower surfaces of the block, and at a right angle to the sides of the block. A knife in this position is illustrated in Fig. 11, A ; it enables ribbons to be cut, and is hence used for nearly all paraffin work, though never for celloidin. But double-embedded material is cut with a square knife.

A *slanting* or *oblique knife* (see Fig. 11, C) is employed in the following instances:

(*a*) For cutting all celloidin material (except when double embedded). Celloidin has a certain elasticity. Unless cut

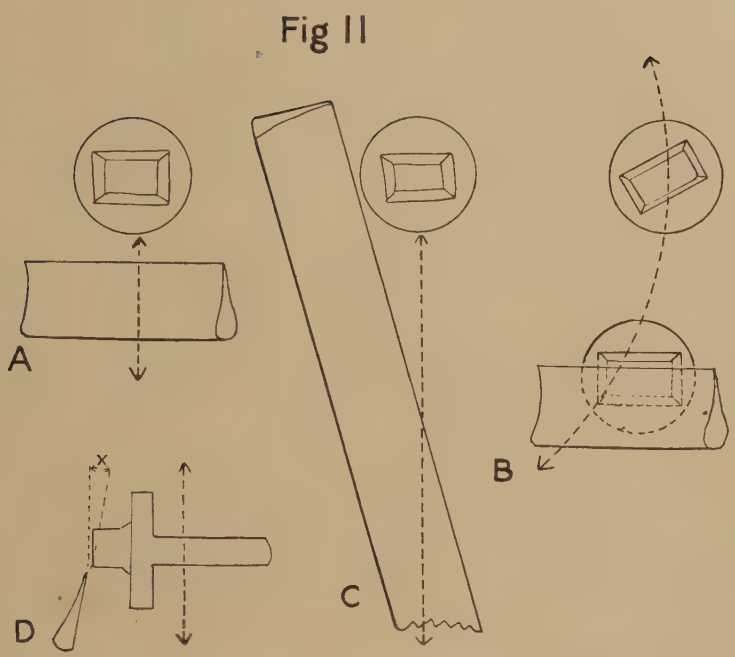


FIG. 11.—ADJUSTMENT OF THE OBJECT IN RELATION TO SLANT AND TILT.

Dotted arrow-lines indicate the motion of the object.

A. The object presents a square face to the knife edge. *Only used for paraffin sections on machines in which the object is raised and lowered at right angles to the knife edge.* (Most rotary machines and the small Cambridge rocker.) The upper and lower surfaces of the paraffin block must be parallel to each other *and* to the knife edge.

B. *The object moves up and down in an arc of a circle which is in the same plane as the knife edge. For paraffin work the object is orientated so that its UPPER surface is parallel with the knife edge at the moment of passing it.* (Large Cambridge rocker and small Spencer rotary microtome.)

C. *A slanting knife. Used for all celloidin work,* whether on rotary or sliding microtomes. Also for paraffin sections on sliding microtomes. In the latter type it is almost always the knife, and not the object, which moves.

D. *The tilt of the knife.* Supposing the object to move vertically in front of the knife edge, angle X is the tilt. With knives of the wedge and plane-concave pattern only a slight tilt is necessary. With biconcave knives a greater tilt is essential if the paraffin block is not to hit against the back of the knife. For further information as to tilt, see p. 74.

obliquely the celloidin is compressed during sectioning, and crumpled sections result.

(*b*) For cutting hard material embedded in paraffin. It is, of course, impossible to obtain ribbons with the knife in this position, but a knife so placed is advantageous in such cases, because the surface of the object in contact with the knife edge is lessened, and the resistance consequently reduced.

(B) Tilt.—Bolles Lee defines the tilt of the knife as "the angle that a plane passing through its back and edge makes with the plane of section." This angle is never more than 90° nor less than 75°. Fig. 11, D, indicates what is meant by the tilt of the knife.

As a general rule it may be remembered that a biconcave knife gives the best results when almost vertical with the knife-holder (*i.e.* when the knife-plane and the section-plane are almost at right angles to one another).

Knives with a plane surface on both sides usually require a greater tilt than this. The angle between knife-plane and section-plane is thus reduced to about 80°. It is necessary to tilt the base of the knife *away* from the object, in order to obviate the latter being compressed against the knife during section cutting.

C. PARAFFIN SECTIONS

For this purpose a microtome with a rapid action—*e.g.* an instrument of the rocker or rotary type—is necessary. The following instructions can only serve as a general guide. They need to be accompanied by actual manipulation of the microtome in order to be of value to the beginner. They apply more especially to microtomes of the rotary or rocker types.

1. **Preparation of the Embedding Mass.**—The tissues are assumed to have been "blocked." It is now necessary to cut them out of the paraffin. Proceed as follows :

(*a*) Orientate the block so that the surface of the object which you wish to section lies face upwards.

(*b*) Trim away the paraffin by downward parings with a scalpel until the object is surrounded by a cube or rectangle of wax. At least 1 mm. of paraffin should be allowed to remain around the object. Until experience has been

gained it is safer to leave more paraffin around the object than this. Note that the block must be trimmed so as to form either a cube or a rectangle. It is especially important to remember that what will be the upper and lower surfaces of the block (in relation to the knife edge when mounted on the microtome) should be parallel to one another.

(*c*) Fix the object on to the object-holder or chuck. Warm the handle of an all-metal scalpel or other convenient instrument in the flame of a Bunsen burner for five or ten seconds. Melt the paraffin on the under surface of the block (*i.e.* the surface opposite the cutting surface). Then quickly press it on to the object-holder. The chuck of a rotary microtome is best supported by inserting the shaft into a small bottle during the application of block to chuck. You can thus hold the block in one hand and the warm scalpel in the other.

2. Preparation of the Microtome.—Screw back the feed mechanism. See that the knife is properly fixed in position. Adjust the indicator regulating the thickness of the sections. Most routine histological work is done with sections of 7 or 8 μ in thickness. For research purposes it is sometimes necessary to cut them as thin as 4 μ, only very rarely less. If the paraffin on the cutting surface of the object has not been trimmed down, begin cutting at 12 to 15 μ, but not more, otherwise the knife edge will be blunted. Then, when the object is fully in contact with the knife edge, reduce the thickness of the sections.

The thickness at which sections are cut is more important than is usually supposed. Apart from the fact that both maximal and minimal thickness are limited by the consistency of the tissues and the embedding mass, there remains the relation of the thickness of the section to the nature of the tissue. Generally speaking, the looser the texture of a tissue or organ (for example, de-fatted adipose tissue or normal lung) the thicker can sections be made. Not only are thick sections of such material easier to cut, but they show more when studied with the microscope than thin ones. Inversely, a dense tissue, packed with cells (such as spleen) is far easier to resolve with the microscope in thin than in thick sections. Or again, while a consolidated lung demands sections of 5 to 10 μ the normal one can be profitably studied at 15 to 40 μ. See also p. 229 in this connection.

It therefore follows that the routine making of sections of all tissues of the same thickness is contra-indicated.

Fix the object-holder in position. All high-grade microtomes have some device whereby the block can be orientated once it has been stuck on to the object-holder. Adjust this so that (*a*) the object will be sectioned in the desired plane, and (*b*) the paraffin block is correctly orientated in its relation to the knife edge.

With microtomes in which the block moves *vertically* before the knife edge, adjust so that both upper and lower surfaces are parallel to the knife edge, as indicated in Fig. 11, A. Such machines comprise the small type of Cambridge rocker and also the majority of rotary microtomes.

With those microtomes in which the object is moved *not* vertically but in the *arc of a circle* in the same plane as the knife edge, it is obvious that upper and lower surfaces cannot both be parallel to the knife edge. In such cases adjust so that the *upper* surface of the object is parallel to the knife edge at the moment when object passes in front of the latter. This is indicated in Fig. 11, B. Some rotary microtomes (*e.g.* the small Spencer model), and the larger patterns of the Cambridge rocker come into this category.

3. Cutting the Sections.—Provided that the above adjustments have been properly made, that the knife is sharp, and that both paraffin and object are of the proper consistency, the sections will adhere to one another to form a ribbon. Within limits, the faster the sections are cut, the better will they adhere together. Each individual section is attached by its top edge to the bottom edge of the succeeding one. Adherence of the sections the one to the other is due to the fact that the impact of the paraffin block against the knife edge generates a little heat.

If the sections will not cut, or section badly, note their behaviour and refer to the paragraph on "Table of Errors in Section Cutting," on p. 81; also to "Additional Notes," p. 82.

4. Manipulation of the Ribbon. — The free end of the ribbon should be gently held with a forceps or supported by

a fine camel's-hair brush moistened with glycerine and albumen solution (see under). Use a brush for serial sections, as a forceps is apt to damage the section held by it. After 8 to 12 inches have been cut, the other end of the ribbon is detached from the microtome knife by inserting a scalpel between it and the knife edge. It is then placed on a piece of paper. The forceps or brush for manipulating the free end of the ribbon is held in the left hand, while the microtome is worked by the right. The scalpel used for detaching the ribbon from the knife should be kept close to the right hand, so that it can be easily picked up by that hand after a sufficient length of ribbon has been cut.

When serial sections are not required, it suffices to stop cutting as soon as the sections pass right through the object. For serial work the ribbons are laid out in parallel lengths on a piece of paper. If the sections are not immediately mounted on slides they should be kept away from dust under a glass dish or special section cover. Never attempt to cut serial sections in a draught, as the ribbons are easily carried away.

5. Fixing Sections on the Slide. — During the process of cutting the sections are always compressed and usually pleated. They need to be flattened out and mounted on slides before they can be stained. There are many ways of doing this, but the following is the simplest:

Preparation of the Adhesive Solution.—Break open an egg and pour the white (but *not* the yolk) into a measure. Then add an equal volume of pure glycerine. Pour into a bowl and mix thoroughly. An egg-whisk is useful in this respect. Then filter through a filter paper in the usual manner. This usually takes several weeks.[1] To the filtrate (which should be of a clear yellow colour) add a crystal of thymol. This will inhibit the growth of bacteria and fungi. The solution will keep for months in a well-stoppered bottle.

To prepare the glycerine and albumen solution for mounting sections, add 3 to 4 drops of the stock mixture to every 15 c.c. of distilled water—*i.e.* 4 drops to a small capsule full of distilled water.

[1] If you filter the egg-white *before* adding the glycerine, the process is more rapid.

Have ready some clean slides (for method of cleaning these, see p. 120), a camel's-hair brush, a scalpel, and a grease pencil or (preferably) a diamond for marking the slides. Proceed as follows:

(i) Cut the ribbon into suitable lengths so that the rows of sections on the slide—when flattened out—will not project when finally mounted beneath a cover-slip. If you do not want serial sections, regulate the length of the strips of sections so that they will lie beneath a square cover-slip ($\frac{7}{8} \times \frac{7}{8}$ inch). For serial sections use longer lengths of ribbon so as to concentrate the maximum number of sections on every slide. For such work long cover-slips ($1\frac{3}{4} \times \frac{7}{8}$ inches or $2 \times \frac{7}{8}$ inches) are employed. Remember that the sections spread out during the flattening process described in paragraph (iii); therefore the length of the cut slips of ribbon should be from two-thirds to three-quarters of the length of the cover-slip which you will put over the sections once they have been stained. Each strip must be of equal length. To attain this end, either count the sections (if they are large), or impale a match stick of suitable length on a mounted needle and use this as a measure when you cut the ribbon into strips.

(ii) Flood the surface of a slide with the adhesive solution. Then pick up the first strip of the series by lightly touching the upper surface of the beginning of it with the brush, which should be moistened with the adhesive solution. Then lower the ribbon on to the slide, placing the blade of a scalpel held flat beneath the other end. Lower first the brush end of the strip on to the fluid, then the scalpel end. In this way you avoid air-bubbles beneath the sections. Repeat the above for every strip. Remember always to make each series of sections begin at the same end of the slide—*e.g.* the bottom left-hand corner of the slide as it lies on the bench in front of you. Number or mark each slide as soon as it has received its complement of sections. It is essential literally to flood the surface of the slide with the adhesive solution so that the sections float freely on the surface of the fluid. Unless this is done they will not flatten out. But avoid getting any of the fluid on top of the sections.

(iii) Flatten out the sections. This can be done over a

hot plate. The latter is made of one or two sheets of superimposed copper beneath which are several small gas burners. The hot plate must be regulated so that its temperature is a few degrees below the melting-point of the paraffin of the sections (*i.e.* 45° to 50° C.). The easiest way for the beginner to ascertain the correct temperature is to lay a thermometer on top of the plate. But with experience the hand is a sufficient guide.

Place the slides upon the hot plate. You will notice that the sections expand under the influence of the heat, thereby flattening out any creases. After you have attained some experience in manipulating sections, by far the simplest way of flattening them is to hold each slide over a Bunsen burner. Note that the sections will be damaged (if not ruined) if you melt the paraffin. Once the sections are flat, hold the slide edgeways and draw it across the finger so as to eliminate the surplus fluid. Next orientate the sections on the slide with a needle or the point of a fine scalpel. If you intend to mount beneath a square cover-slip, arrange the sections in the centre of the slide. But if you intend to mount under a long cover-slip (as for serial sections), leave a clear space of $\frac{3}{4}$ to 1 inch at one end of the slide—this allows room for a label—and at least $\frac{1}{4}$ inch at the other end. Never arrange sections so that they lie right at the edge of one end of the slide. This is bad: firstly, because when you ultimately look at the sections at the extreme end of the slide under the microscope, you will find them continually going out of focus as you move the slide to and fro. This is due to one end of the slide lying unsupported above the circular hole in the stage. Secondly, sections are often packed for transit by putting small strips of cardboard at each end of the slide. The slides are then packed into little piles and tightly tied together. If the cover-slip is right at one end of the slide it will be pressed upon, and probably broken.

(iv) Dry the slides by leaving them for some time on the hot plate. This is very important for the following reason. The effect of the heat is to coagulate the albumen of the adhesive fluid, thereby causing the sections to adhere firmly to the slide once the water has evaporated. If every trace of

water has not been driven off from the under surface of the sections, these will float off the slide during the subsequent staining and other treatment. The time required for drying slides varies — (*a*) according to the size of the sections; obviously it takes longer to drive away the water from the under surface of a large section than a small one. (*b*) The subsequent treatment of the sections; *i.e.* if you are going to stain in watery solutions for many hours, the drying must be prolonged; otherwise the sections will float off the slide. (*c*) The nature of the material. Dense tissues—and especially sections of the central nervous system — require careful drying.

The minimum time required for drying can only be learned by experience. The following indications will form a rough-and-ready guide. They apply to sections dried on the hot plate at a temperature of 48° to 50° C. Medium-sized sections, provided prolonged immersion in watery stains be avoided, can be dried in half to one hour. If prolonged treatment with aqueous media be contemplated, the time should be increased to two or three hours. Very large sections should be left to dry overnight. So also should all material fixed in chrome-osmium or formol-bichromate mixtures. For tissues thus treated are liable subsequently to become detached from the slide. Sections of nervous system also need several hours, or, preferably, overnight treatment on the hot plate. For research purposes it is customary to dry the sections overnight.

An ordinary biological incubator, kept at 37° C., makes an excellent drying chamber. Flatten out the sections over the Bunsen burner, then leave the slides overnight in the incubator. In warm and very dry climates sections can be allowed to dry in the air away from dust. This requires twelve to twenty-four hours.

While under-drying is a mistake, no harm is done to slides even if they be left for days on the hot plate, provided that the paraffin is not melted and that the sections are protected from dust.

When the sections are dry, they can be stained, for the general technique of which see Chapter IX.

6. Table of Errors in Section Cutting, and their Remedy

Fault.	Cause.	Remedy.
Ribbon is curved instead of straight.	*Either* the upper and lower surfaces of the block are not parallel to one another, *or* (if one border of the sections is crumpled) the knife edge is dull at that point.	(*a*) Pare away the paraffin from block until upper and lower surfaces are parallel. (*b*) Move the knife along the holder so that the knife edge presents a uniformly sharp edge to the block.
Ribbon curls upwards as sections are cut, so that they get broken against knife edge.	The "tilt" of the knife is too great.	Adjust knife so as to bring it nearer to the vertical.
Sections cut well individually but do not adhere together to form a ribbon.	The paraffin is too hard for the temperature at which the sections are being cut.	(*a*) Coat the upper surface of the block with a layer of soft (45° C.) paraffin. (*b*) Warm the room or place a Bunsen burner very close to the microtome. (*c*) Cut the sections rapidly.
Sections crumple.	The paraffin is too soft *or* the knife is blunt.	(*a*) Cool the block in water. (*b*) Sharpen the knife.
Sections are longitudinally scratched.	(*a*) Knife edge is serrated. (*b*) Knife edge is coated with débris from the sections. (A tiny hairlet on the knife edge will split thin sections.)	(*a*) Remove teeth on hone. (*b*) Clean knife edge by passing it between finger and thumb.
Sections cut sometimes thick, sometimes thin.	(*a*) Feed mechanism is badly adjusted. (*b*) Knife improperly clamped. (*c*) Object-holder is loose.	(*a*), (*b*), (*c*) } Obvious.

Fault.	Cause.	Remedy.
There are thick and thin zones in each section.	The knife edge vibrates owing to— (*a*) hardness of object, (*b*) excessive tilt of knife.	(*a*) Collodionise the sections. (*b*) Reduce tilt to minimum.
Resistance is felt when block is near downward limit of its travel.	Tilt of knife is insufficient. Paraffin block is therefore compressed against base of knife towards end of stroke.	Increase tilt.
Sections of unequal thickness are produced. During impact of object against knife edge a harsh metallic note is emitted.	Tilt of knife is too great. The object is compressed during sectioning against the knife edge. This is proved by the fact that the knife will cut a second section (after feed mechanism has been thrown out) owing to the block recovering its elasticity.	Reduce tilt.

As a last resort cut the sections on a sliding microtome and collodionise each one. See next page.

7. Additional Notes on Paraffin Section Cutting

Flattening out Creased Sections.—Sometimes the method outlined above does not suffice. In such cases pull out each end of the section (after preliminary warming) with needles. As a last resort, slightly melt the paraffin. Avoid doing this unless you have to, because melting the paraffin—until the sections have adhered to the slide after drying—causes artificial spaces between the tissue components to be formed, while pre-existing ones are exaggerated. Also, sections, the paraffin of which has been melted before adhesion, are apt to become detached from the slide during staining.

Another useful method, *not applicable to serial sections*, is the following: Heat a saucepanful of water to a temperature just below the melting-point of the paraffin used for embedding. Drop short segments of the ribbon on to the water. Then float the segments on to slides. To cause adhesion of the sections, place a tiny drop of the concentrated glycerine and albumen mixture (about the size of a pin's head) on each slide; spread the drop with the finger; then float the section on to the slide. Dry the sections in the usual way.

Collodionisation.—If the object is so brittle that all the sections are broken, the following method is often of great service:

Fix the block on to the sliding microtome. Have ready a capsule of glycerine and albumen solution and a camel's-hair brush. Also a 0·5 per cent. to 1 per cent. solution of celloidin in absolute alcohol and ether. Another camel's-hair brush, stuck through a square of tin or cardboard, is placed in the celloidin solution so that the brush dips into it.

You proceed thus:

(1) Wipe the cutting surface of the block with the celloidin solution. The brush should be drained against the neck of the bottle and quickly passed once over the cutting face of the block.

(2) Allow two to three seconds for the celloidin to dry, and cut the section.

(3) Pick up the section with the other brush, moistened in the adhesive solution, and place it on a slide flooded with the adhesive solution.

(4) Repeat processes 1, 2, and 3.

Notes.—Serial sections may be cut by this method, but it requires experience.

The sections require careful flattening out on account of the celloidin layer, which tends to inhibit this unless the paraffin is warmed to just beneath its melting-point.

Distinguishing between Upper and Lower Surfaces of Sections.—Sometimes a section, or whole ribbon, is accidentally turned over during its manipulation. A slight draught, or even the breath, may cause this, as the result of which the upper and lower portions of the section or sections become

inverted. It is important to avoid mounting sections "the wrong way round" in serial work. Remember that the lower surface of a paraffin section is always the shinier.

Compression of Paraffin Block during Section Cutting.—The reiterated impact of the knife edge against the block causes the latter slightly to contract. If you cease cutting a block when about half-way through, and then return to it some time later, you will notice that the first section which comes off will be many times the thickness of those which you had previously cut. This is simply due to the expansion of the paraffin block when freed from the wedge-like pressure of the knife edge on it. It is therefore necessary in serial work slightly to screw back the feed mechanism if you cease cutting the sections before you have cut right through the block.

Loss of Sections from a Series.—For ordinary purposes a break in the continuity of the rows of sections on the slide is of no importance. In serial work it is another matter. If you lose or destroy one (or more) sections from a series, leave a space of one (or more) sections on the slide between the preceding and succeeding sections. The observance of this rule is especially important in embryological research work. Nothing is more misleading than "serial" sections not in a series, though, to the practised eye, the gap is often evident.

D. CELLOIDIN SECTIONS

As already explained, an oblique knife is essential. Although most celloidin work is accomplished with the sliding microtome, quite good results can be obtained with a rotary machine fitted with a special (oblique) knife-holder. The celloidin block (after trimming) is attached to a vulcanite block as already explained on p. 62. The block is then inserted in the microtome. The cutting movement should be slow and shearing.

1. Sectioning by the "Wet" Method.—Material embedded by the ordinary ("wet") method must be cut "wet"—*i.e.* both knife and object are moistened with alcohol of 70 per cent. As each section is cut it is transferred to some 70 per

cent. alcohol in a watch-glass or small capsule by means of a brush moistened in the alcohol. Sections can be preserved indefinitely in 70 per cent. alcohol until stained and mounted (for the technique of which see p. 146).

2. Sectioning by the "Dry" Method.—Objects embedded by Lee's method (often known as the "dry" method) can be cut dry. This is a great advantage. Owing to the infiltration of the tissue with the oil of cedar, the cutting surface remains sufficiently moist to enable excellent sections to be made without the tiresome necessity of pouring alcohol on knife and object. The sections are preserved in 70 per cent. alcohol until required.

3. Fixing Celloidin Sections on the Slide. — Celloidin sections when made by the wet or dry methods are usually stained in watch-glasses. They are not fixed on to slides until finally mounted in balsam. Sometimes it is more convenient to stain and otherwise manipulate celloidin sections on the slide. The following is the simplest way of attaching celloidin sections to the slide :

(1) Place the sections in 95 per cent. alcohol for a few minutes.

(2) Arrange them on the slide.

(3) Pour *ether vapour* over the sections. To do this, put a little ether in a bottle and shake. Then hold the bottle aslant over the sections. But avoid pouring any fluid ether over them. The sections will become transparent. Then,

(4) Place the slide in 80 per cent. alcohol for ten minutes.

NOTE.—The ether vapour causes the sections to adhere to the slide because of its solvent action on celloidin. Therefore treat the sections for the minimum time with the vapour. The slide is subsequently transferred to the 80 per cent. alcohol so as to harden the celloidin.

For valuable (*e.g.* serial) material, many workers prefer the slow but safe watch-glass method.

Sections of material which has been double-embedded are attached to slides by the glycerine and albumen method just as for ordinary paraffin sections. But care is needed in the flattening out, which is sometimes difficult.

E. PARAFFIN-CELLOIDIN SECTIONS

Tissues which have been double-embedded by the celloidin-paraffin technique are cut on the rotary microtome as for ordinary paraffin material. A horizontal (not a slanting) knife is employed, and serial sections in the form of ribbons can be obtained with this method.

F. FROZEN SECTIONS

1. **Preparatory Treatment.**—The fixative, *par excellence*, for making sections of frozen material, is 4 *or* 5 *per cent. formol* in isotonic saline. Not only does this enable you to apply the standard fat stains, certain special methods for the central nervous system, as well as general histological stains, but it gives to tissues a degree of consistency peculiarly favourable to the freezing microtome. Sections from 5 μ upwards can be obtained.

Standard Technique.—Fix small pieces of tissue for twelve to twenty-four hours in formol. For rapid work fix thin slices in this medium at 37° or 40° C. for ten minutes.

Good sections are obtained by cutting the tissues direct from formol. They may also be sectioned after a few hours' washing in running water.

The following considerations must be taken into account when you wish to section other than formol-fixed material.

Mercuric Chloride.—Apt to overharden tissues for the freezing method. Wash out $HgCl_2$ deposit with iodine alcohol; then rinse in running water for some hours before sectioning.

The Fixatives of Flemming and of Champy, and other chrome-osmium mixtures, require the usual washing in water, followed by a bath of 50 per cent. alcohol and another rinsing in water.

Alcohol-preserved tissues should have several hours washing in running water, as alcohol in the tissues inhibits freezing. Kaiserling's solution—the common preserving medium of the morbid anatomist for naked-eye specimens—requires three to six hours' washing out in running water.

Remember that the use of warm water (*circa* 37° C.) greatly accelerates the washing out of the above fixatives.

Inclusion in Gelatine.—This method is due to Gaskell and Graff. The modification described below is that of Nicolas.

Gelatine embedding is indicated for tissues of a loose texture. Transverse sections of a papilloma, for instance, are held together by this method when they would break up by the ordinary freezing technique.

Proceed as follows :

1. Fix—or preserve—in 5 per cent. formol.
2. Rinse the piece of tissue, after trimming it, in running water for twenty-four hours.
3. Soak the object in a 12·5 per cent. solution of gelatine at 37° C. for three to twenty-four hours.
4. Treat similarly in a 25 per cent. solution of gelatine.
5. Embed in a paper box in a very thick warm gelatine solution. Allow to cool. [I find the 25 per cent. solution quite thick enough.]
6. Harden the gelatine in the standard 4 or 5 per cent. formol solution for an hour or two.
7. Wash out the formol from the gelatine block by running water for an hour or two.
8. Trim the block and freeze it hard. Make the sections when it has sufficiently warmed to become soft.

Notes.—(1) The principle of hardening the gelatine is its conversion from the hydrosol to the hydrogel condition already described on p. 15.

(2) The gelatine investment of the sections is retained during staining. It holds the tissues together, while it absorbs dyes only very slightly.

(3) Gelatine-permeated tissues offer some resistance to freezing, whence the need for over-freezing in order to make sure that the central portion of the block has been attained.

2. Section Cutting. — With the freezing microtome sections from 5 μ upwards can be cut without any previous treatment beyond fixation in formol. It is chiefly used in histological and pathological laboratories for the following purposes :

(i) For the identification of fat.

(ii) For certain impregnation methods for the central nervous system.

(iii) For the rapid preparation of microscopical sections. Sometimes this is made use of by the surgeon, since fragments of a tumour may be stained, examined, and a diagnosis made, in a few minutes from the moment of their excision.

An important advantage, furthermore, of the freezing technique is that less shrinkage is inflicted on tissues than by the paraffin method of inclusion. Comparison of frozen and paraffin sections of the same organ is very instructive in this regard, and shows indisputably that no matter how carefully tissues be passed through different grades of alcohol, of alcohol plus the clearing medium, and of the clearing medium plus paraffin, shrinkage is inevitable.

Against the freezing method is the extreme difficulty of obtaining anything approaching serial sections; further, such sections are free and have to be stained in watch-glasses or capsules. In such respects the frozen sections compare unfavourably with paraffin ones, which can be stored on the slide for years before staining, and, when stained, are easily and rapidly handled.

The Freezing Microtome.—The principle is the same as in the paraffin or celloidin microtomes: the object is raised a definite number of micra with each stroke of the knife. A knife with plane surfaces—*i.e.* neither biconcave nor plano-concave—is a necessity. The object is frozen until of a consistency sufficient to enable thin sections to be made. The consistency imparted to the tissues by being just frozen is such that a medium of inclusion is unnecessary. Only for certain difficult objects—*e.g.* a papilloma in transverse section—is it advisable to have recourse to the gelatine method of inclusion.

Types of Freezing Microtome.—There are two main types of freezing microtome: the ether and the carbon-dioxide machines.

In the ether microtome the requisite chilling of the object is produced by the vaporisation of ether in or around the object-holder. Only small objects can be frozen by this method, which has now given way to the far more efficient

carbon-dioxide instrument. The chuck of this machine, as shown in Fig. 12B, is hollow and bordered peripherally by large holes. CO_2 is led into this from a cylinder in the shape

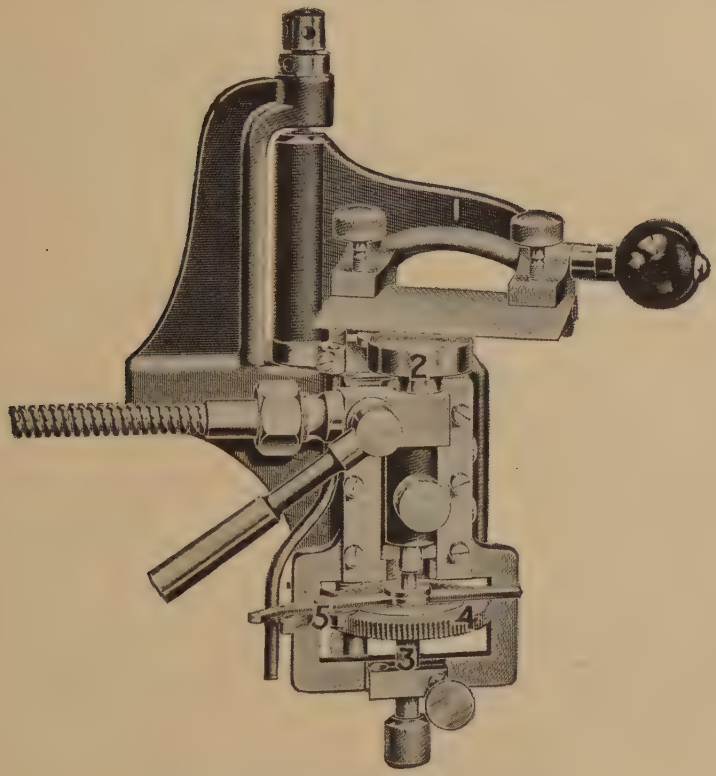


FIG. 12A.—FREEZING MICROTOME OF LEITZ.

This nstrument, especially suitable for frozen sections, is clamped to the bench. The knife is mounted on an arm (1), the object on a vertical chuck (2). There is the ordinary arrangement of micrometer screw (3), ratchet wheel (4), and pawl (5) for the automatic raising of the object. The holes in the chuck, through which the CO_2 issues, are shown in Fig. 12B.

of fine carbon-dioxide snow, the evaporation of which freezes the object. In modern carbon-dioxide microtomes there exists the usual mechanism whereby the object is raised the proper number of micra after each stroke of the knife (Fig. 12A).

The connection between microtome and carbon-dioxide

cylinder is important. To obtain the best results the following conditions must be fulfilled :

1. The connecting tube between cylinder and chuck should be as short as possible, preferably 20 to 25 cm. (8 to 12 inches).

2. The tubing should be of metal, and its internal bore should be very fine (2 mm.).

3. The CO_2 cylinder must be attached by a bracket to the microtome bench so that its valve is upside down.

When the microtome is so installed, any water in the cylinder

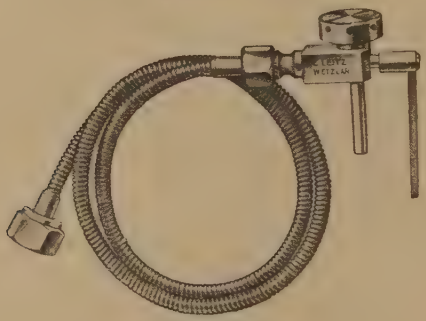


Fig. 12B.

Illustrating the metal tube and hollow chuck with valve for use in the CO_2 microtome depicted in Fig. 12A. The tube is of very narrow bore and opens beneath the chuck by a tiny aperture. In this way the maximum of decompression of the gas is assured. Outside the copper tube is a flexible guard tube as shown in the figure.

is promptly blown out of the chuck, while a short solid metal pipe renders remote the possibility of its blockage by freezing in one part. Remember that CO_2 is stored in cylinders at a pressure of 1000 to 1400 lb. per square inch. Should the tube freeze close to the chuck a considerable pressure is rapidly made up in it, and, unless the tube is of good quality brass or copper, it may burst. The writer once nearly witnessed serious damage being inflicted to the face of a laboratory worker by the bursting of a tube owing to blocking by freezing of its distal extremity and freezing of the valve of the cylinder. A long tube and a cylinder kept valve uppermost were responsible for this.

Section Cutting with the Freezing Microtome.—Only the CO_2 type is described here. The machine should be well oiled with a thin oil before use so as to avoid uneven motion. The *modus operandi* is as follows :

1. Place the object on the chuck in a drop of water. Its thickness should not be more than 3 mm., otherwise you will have difficulty in freezing it evenly.

2. Open slightly the valve of the cylinder, then open the valve on the chuck for a moment once every few seconds. Repeat the process as often as necessary. When the water on the chuck has turned to ice—

3. Quickly make the sections. (Usually one has to wait a short time till the tissues are beginning to thaw.)

4. Transfer them with a moistened camel's-hair brush to a large shallow capsule of distilled water or 50 per cent. alcohol. (The general procedure for staining the sections is to be found on p. 149 ; the special methods for fat and the central nervous system on pp. 167 and 233 respectively.)

Notes.—(1) If the object is insufficiently frozen, the sections crumble and the block of tissue becomes detached from the chuck.

(2) If over-frozen, it is so hard that it will not cut. In this case wait a few moments for it to soften, or accelerate the process by putting the finger on it.

(3) The knife must be kept dry.

(4) Some objects only adhere with difficulty to the chuck. In such instances, place a layer of thin wet gauze between object and chuck (Schmorl).

(5) Move up the object very gradually towards the knife before making the first section, as contact with the knife edge with too great a thickness of the object (especially if this be over-frozen) will blunt the edge of the knife.

(6) If the carbon-dioxide cylinder is new, it is safer to disconnect the pipe from the chuck and blow off for five to ten seconds before re-connecting. The idea of this is to blow off any water from the cylinder.

(7) Keep the cylinder protected from strong sunlight or heat. It is safer.

CHAPTER VII

GENERAL OUTLINE OF THE THEORY AND PRACTICE OF STAINING

(IN COLLABORATION WITH F. HAYNES)

1. Historical—2. Substances employed for Staining—3. Staining and Impregnation—4. Basic, Acid, and Neutral Dyes—5. General Technique of Staining Fixed Tissues — 6. Metachromasy — 7. Specific Stains—8. Theories of Staining.

INTRODUCTORY

THE aim of this chapter is to supply an outline of the principles of staining, and thus to serve as a basis for the succeeding chapters which deal with the technique of the staining processes in histology. Unfortunately the reason why certain tissue constituents have an affinity for one dye and not for another is still far from clear.

1. **Historical.**—The foundations of microscopic anatomy were laid before the advent of staining methods. Tissues were dissociated in various colourless media, or else roughly sectioned by hand, and examined between slide and coverslip. Although the methods were crude, prolonged and accurate observation of their material enabled the histologists of the earlier half and middle of the nineteenth century to describe the arrangement of the cells and tissues in the various organs of the body. That they were able to accomplish this with remarkable accuracy is evidenced by reference to such a work as Kölliker's *Microscopic Anatomy*, published in 1852. The study of—and even the possibility of seeing—certain cell and tissue components was impossible until the advent of staining methods, and, had not these been evolved, we should know little or nothing regarding the mutual relationship of the cells and fibres of the central nervous system, the

structure of the organs of special sense, or the components of the cell. Cytology, in particular, only came into existence when the introduction of certain stains made it possible selectively to stain certain elements of the cell such as the chromosomes, the mitochondria, and the Golgi bodies.

Carmine staining was first introduced by the botanists Goppert and Cohn in 1849 ; hæmatoxylin in watery extract in 1863 by Waldeyer, and the aniline dyes by various observers somewhat later. It is especially to Ehrlich, Flemming, and Unna that the invention of rational formulæ for both aniline and hæmatoxylin series is due, while Martin Heidenhain, in 1891, introduced the iron-hæmatoxylin method, which still remains the standard technique of the cytologist.

Most present-day histology is done with the aid of the hæmatoxylin and aniline dyes, only rarely with the carmine stains, though these are still retained by the zoologist for the staining of " whole preparations " (*i.e.* entire organisms) such as Cestodes, Medusæ, etc.

2. Substances employed for Staining by the Histologist.—The majority of the substances employed for the differential staining of cells and tissues are solutions of various coloured compounds (frequently used for dyeing in the textile industry). These substances comprise :

(i) *The Extracts from Certain Animals and Plants.*—Examples of this are carmine—an extract from the females of the insect *Coccus cacti coccinillifera*—and hæmatoxylin, extracted from the bark of a tree, *Hæmatoxylon campechianum.*

(ii) *The Vast Group of the Aniline Dyes,* prepared from aniline and its homologues (toluidin, etc.).

3. Staining and Impregnation.—All the above substances are *stains,* in the sense that the cell and tissue components, when properly treated, take up the colouring matter from the solution. No solid dye particles can be seen in the stained elements, which, unless very darkly stained, retain their transparency. Roughly stated, this is because the stain is held in a molecular state by the tissues.

For certain purposes, and especially for the bringing into evidence of the cells and their processes of the central nervous system, use is made of the method of *Impregnation.* Some

substances, notably the salts of the heavy metals, although colourless in solution, are selectively precipitated over certain cell and tissue components. An impregnation is usually regarded as differing from a stain in that (i) it is opaque, (ii) it is particulate, and (iii) it is deposited *around* the elements which it has brought into evidence.

Silver nitrate ($AgNO_3$)—the most commonly used impregnating substance—while illustrating these conditions, also shows that (in certain cases) a genuine transparent and non-particulate stain of cells may result. Consequently, the above distinctions between impregnation and staining are only approximate, and impregnations, under certain conditions, become true stains.

The chief difference between staining and impregnation lies in the fact that stains are coloured in solution while impregnating reagents are not. A stain is taken up by the tissue, while the impregnating reagent is broken down by contact with the tissue, usually to a lower oxide or to the pure metal.

4. Basic, Acid, and Neutral Dyes.—The vast majority of the stains employed in histology are *salts*.

By a basic stain is meant a dye (*i.e.* a salt), the base of which contains the colouring substance, combined with an acid—usually inorganic. The acid component of such a dye molecule is usually hydrochloric, sulphuric, or acetic acid. Thus, *basic fuchsin* is composed of the base rosaniline (coloured) combined with the acid radicle HCl (colourless).

An acid stain is one in which the acid component of the dye molecule is the coloured one, the basic component being colourless. The base is usually sodium or some other inorganic radicle. For instance, *acid fuchsin* is the sodium salt of a sulphonic acid derivative of rosaniline.

A neutral dye is formed when aqueous solutions of certain basic and acid dyes are mixed. Owing to the interaction of already large dye molecules, solutions of neutral stains are often colloidal.

Neutral dyes are soluble in alcohol, rarely so in water. Basic and acid dyes are soluble sometimes in water, sometimes in alcohol, very frequently in both.

5. General Technique of Staining Fixed Tissues.—The

stains we employ may be conveniently grouped—as already done for fixatives—into (i) micro-anatomical and (ii) cytological stains.

A micro-anatomical stain is one which, while staining tissues (*i.e.* aggregates of cells) in a demonstrative manner, does not pick out the details of the cells themselves. The majority of the carmine stains, and Ehrlich's and Delafield's hæmatoxylin, are examples of micro-anatomical stains.

A cytological stain is one which demonstrates certain elements, nuclear or cytoplasmic, of the cells without necessarily aiding in the differentiation of the various tissues. Methyl-green, which has a special affinity for chromatin, and Best's carmine stain for glycogen, are respectively examples of cytological nuclear and cytoplasmic stains.

As with the classification of fixatives, so with this scheme of stains—there is a good deal of overlap. Thus, Heidenhain's iron-hæmatoxylin is one of the best stains for demonstrating nuclear and cytoplasmic elements, although only rarely can both be equally well stained in one and the same section.

And, finally, we must add to the classification outlined above, under the heading of "Cytological Stains," the group of intravitam dyes, which are applied to living (and consequently unfixed) cells.

The following scheme illustrates the above remarks :

Staining Methods	(i) Micro-anatomical		Applicable (with rare exceptions) to fixed material only.
	(ii) Cytological	Nuclear	
		Cytoplasmic	
	(iii) Intravitam (= vital staining)		Applied only to living cells and tissues.

Certain terms, largely derived from the dyeing of textiles, and in common use in histological technique, must now be noted.

Progressive and Regressive Stains.—*A progressive stain* is one which slowly stains the elements of tissues in a definite sequence. Thus, certain hæmatoxylin stains, *e.g.* Delafield's or Ehrlich's, and many of the carmine dyes, stain first of all the nuclei, and then the cytoplasm of the cell. By taking the smear or section out of the staining bath at the

right moment, either the nuclei alone, or the nuclei *and* the cytoplasm, will be stained as desired. *A regressive stain* is one which involves, first the overstaining of the material (usually till all histological detail is obliterated), and then the washing out of the stain until it is retained only by the cell or tissue components which are to be studied. The second stage of the regressive method is known as "*destaining*" or "*differentiation.*"

Regressive staining is far more commonly employed than the older progressive method. Practically all the aniline dyes are differentiated, so are most of the hæmatoxylin series

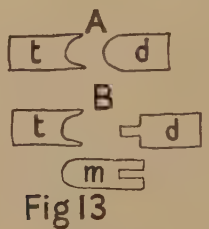


FIG. 13.—ILLUSTRATING STAINING.

A schematises direct staining without the use of a mordant. The tissue (*t*) and the dye molecule (*d*) associate directly by chemical or physical means.

B illustrates the function of the mordant in indirect staining. For *t* and *d* to combine the intervention of *m*, the mordant, is essential. Upon the combination of the mordant with the tissue, staining occurs.

of stains. Delafield's or Ehrlich's hæmatoxylins, often used progressively for the sake of speed in routine histological and pathological work, give a more precise stain for nuclei if the material be first overstained and then differentiated.

DIRECT AND INDIRECT STAINING.—Many of the aniline dyes (*e.g.* methylene-blue or eosin) stain tissues perfectly if the tissues be placed in a simple solution in water or in alcohol of the dye-stuff. This is known as *Direct* (or *Substantive*) *Staining.* Many stains, and especially those of the hæmatoxylin series, necessitate the use of a *mordant.* A mordant has already been described as a substance which makes possible, or facilitates, a particular staining reaction. This it does by combining with the tissue on the one hand,

and the stain on the other. The coloured substance deposited in the tissues is known as a lake—a term derived from the textile industry (see Fig. 13). The mordant may be either combined with the staining solution (*e.g.* the potash alum in Ehrlich's hæmatoxylin) or it may be a separate solution, through which the tissues are passed before being placed in the stain itself (*e.g.* the iron-alum bath of Heidenhain's hæmatoxylin).

Accentuators. — These are substances which, while favouring the action of stains on tissues, do not participate —as do the mordants—in the fixation of the stain by the tissue components. The accentuator plays a part in the staining process which is analogous to catalysis in chemical combination, in that small amounts of these substances greatly increase the staining power and selectivity of the stain without (apparently) any chemical combination on the part of the accentuator.

The most important accentuators comprise :

Substance.	Formula.	Example of Staining Solutions containing it.
Potassium hydroxide	KOH.	Löffler's methylene-blue.
Borax . . .	$Na_2B_2O_7$.	Borax methylene-blue.
Aniline . . .	$C_6H_5NH_2$.	Safranin, gentian violet, and many other synthetic dyes.
Phenol . . .	C_6H_5OH.	Carbol thionin.

The accentuators employed for the impregnation of the central nervous system by silver and gold methods are often hypnotics. Why this should be we do not know. These substances are sometimes termed *Accelerators* (Cajal).

They comprise :

Chloral hydrate Veronal . . Sulphonal . Trional . . Hedonal . Nicotine, etc. .	Employed in the metallic impregnation methods (of Cajal and others) for nerve fibres, etc., in the central nervous system.

6. **Metachromasy.**—Most staining substances stain the

elements of cells and tissues in varying degrees of intensity of the same fundamental colour. Thus, all the structure stained in a section by acid or basic fuchsin will be in shades of red, or, those which have taken up methyl-green or orange G, will be green and orange respectively.

Certain tissues and cell components, however, when treated by certain stains give a metachromatic reaction, in the sense that they do not stain in the fundamental colour of the stain but in another one. For instance, thionin is a blue dye which stains most of the tissue constituents in shades of blue, *except* the ground substance of cartilage, mucin, and mast-cell granules, which are stained *purple*. Similarly, safranin stains the ground substance of cartilage yellow. Another example of metachromasy, made use of by the pathologist, is that of methyl-violet, which stains red not only cartilage, matrix, mucin, and mast-cell granules, but also the morbid infiltration —known as *Amyloid*. Metachromasy appears to be a product of the basic aniline dyes only.

Genuine metachromasy is *not* due to impurities in the stain (*i.e.* the presence of two dye-stuffs).

7. Specific Stains. — Certain constituents of cells and tissues stain only with certain stains which have little or no affinity for other constituents. Such staining methods are said to be " specific " for the constituents in question. It must be recognised, however, that the specificity of stains is largely approximative, and that the attainment of such a reaction usually involves the rigid application of a given method. That is to say, that many of the specific stains are only specific under certain conditions. Some examples (arranged in degree of decreasing specificity) are :

Weigert's elastin stain, which stains elastic fibres blue to black—a truly specific stain.

Scharlach R and Sudan III., which stain intracellular fat red. Specific with the appropriate technique.

Methyl-green, staining chromatin green in presence of acids —fairly trustworthy with appropriate technique. But it gives a positive reaction with certain secretions, notably *mucin* and *silk*.

Janus green (Janus Green B of Hoechst) stains very

selectively the mitochondria intravitam under appropriate conditions.

Van Gieson's stain, staining collagen fibres red—fairly specific.

8. Theories of Staining.—This, the most complex problem of histological technique, can only be cursorily discussed in a précis of this nature. The subject is rendered especially complicated by the fact that staining is due to factors of a chemical, physical, or (usually) physico-chemical nature. For not only is the physical chemistry of cell and tissue components still largely in the making, but the chemical constitution of certain histological dyes—apart from the synthetic series—has yet to be determined.

Although the staining of protoplasm is very controversial and involved, certain facts stand out clearly :

1. Protoplasm, once it has been fixed, often stains in a very selective fashion.

2. Living protoplasm, owing to the controlled permeability of the surface membrane, is very difficult to stain.

3. Dead, but *un*-fixed, protoplasm takes up certain dyes, but its staining reactions are different from 1 and 2.

The discussion of the staining problem is simplified by dealing with the staining of fixed and living protoplasm apart, while the question of the reaction to dye-stuffs of dead but unfixed protoplasm though extremely interesting, is not sufficiently elucidated to warrant further discussion.

A. The Staining of Fixed Protoplasm.—The theories of the staining of fixed protoplasm may be grouped under two headings—(i) chemical ; (ii) physical.

(i) Chemical Conception of Staining.—The partisans of this theory (Ehrlich, Heidenhain, Giemsa, Unna, and many others) hold that stains are retained by certain cell or tissue constituents because of actual chemical combination between the dye and proteid (or other) molecules. The role of fixation—in so far as it modifies the chemical nature of the cell constituents—is regarded as comparatively slight.

Methyl-green, for instance, has marked affinity for the chromatin of both fixed and fresh cells (particularly for the latter). This is said to occur because the methyl-green com-

bines with the chromatin. Again, Giemsa has shown that the basic aniline dyes and metaphosphoric acid furnish precipitates of stained metaphosphates *in vitro*. The same dyes do not give such a precipitate with ortho- or pyrophosphoric acids. Now the cell nucleus is especially rich in phosphorised compounds—and especially in metaphosphoric acid—whence its affinity for the basic aniline dyes. Certain elements of the cell, other than chromatin, have a marked affinity for the basic aniline dyes, *e.g.* the conspicuous cytoplasmic granules of the mast cell. This affinity for the basic dyes is due, according to Unna, to the mast-cell granules being genuinely acid, while, inversely, the affinity of the granules of the eosinophil leucocyte for the acid dyes (eosin, orange G, etc.) is due to the basic nature of these granules.

Ehrlich, who may be looked upon as the originator of the chemical theory of staining, regarded the chromophility of the nuclear and cytoplasmic elements as definitely due to their facility for combination with basic and acid dyes respectively. Ehrlich's views, however, were based on cover-slip preparations of blood cells, fixed (*i.e.* coagulated) by heat. Consequently the influence—chemical or otherwise—of the fixative did not enter into the matter.

We now know that the colour-response of tissues to stains is very largely influenced by fixation. As already explained (p. 96), certain fixatives act as mordants towards certain stains. Acid dyes do not selectively stain only the cytoplasmic structures, nor basic dyes only the nuclear components. Further, the same stain sometimes has affinity for different cell constituents of a different chemical nature. Hæmatoxylin is an acid dye, yet if combined with a mordant (alum) it stains nuclei rather than the cytoplasm. Acid fuchsin is employed as a cytoplasmic stain, but if the section be overstained and then carefully differentiated, it is possible to attain nuclear in lieu of cytoplasmic staining. And, finally, the following observation of Michaelis is against the view that staining is always of the nature of a chemical combination : were staining purely chemical one would expect definite quantitative relations between the staining, and the stainable, substances to be fulfilled. But they are not.

These instances could be multiplied, but they show that while *certain* staining reactions — *e.g.* methyl-green and chromatin, or the basic dyes and mast-cell granules—are probably cases of genuine chemical combination, much—if not most—staining is due to factors other than purely chemical ones.

(ii) Physical Theories of Staining. — A. Fischer, Bayliss, and others have invoked the action of various factors here.

(*a*) *Solubility.*—Sometimes staining is purely a matter of solubility of the dye in the elements of the cell. The staining of intracellular fat with the specific stains Sudan III. and Scharlach R is due to the greater solubility of the dye molecules in the fat as compared to the solvent (70 per cent. alcohol, or a mixture of this and acetone). Such cases, however, are very rare.

(*b*) *Adsorption.*—Adsorption is the property of solid bodies especially when in a state of fine division, to attract and to condense liquid and gaseous substances on their free surfaces. The best known instance of adsorption—partly on account of its industrial applications—is afforded by charcoal. Not only does this substance energetically adsorb gases—a cylinder filled with it can be filled, at a given pressure, with more volumes of a gas than when empty—but it also adsorbs coloured solutions. In addition to molecules, larger particles —colloidal—or even micro-organisms are capable of being adsorbed. The "filtration" of bacterially infected water through the Berkefeld filter is largely an adsorption process, since most bacteria are smaller than the channels of the filter.

Adsorption is often accompanied by electrical phenomena. Thus, substances bearing an electro-positive charge adsorb bodies bearing an electro-negative one, while those bearing negative charges adsorb bodies bearing positive charges.

Now it has been suggested by Bayliss and others that this has a very important bearing on histological staining, since the stainable bodies in the cell are chiefly colloids, and, furthermore, colloids bearing electro-negative charges. Whence their adsorption of the basic dyes—which are mostly electro-positive.

Hæmoglobin, on the other hand, is an electro-positive colloid (which is rare). Whence its adsorption for acid dyes—which are electro-negative.

This hypothesis, however, does not appear to take into consideration the fact that *fixed* protoplasm is no longer colloidal. It is therefore difficult to see how electrical adsorption can be applied to much of the material studied by the histologist.

Until the problem of staining has been attacked by the biochemists and histologists working in conjunction, the *raison d'être* for most staining reactions must be acknowledged to be unknown—or, at any rate, unproven.

CHAPTER VIII

EQUIPMENT FOR STAINING AND FORMULÆ OF TYPE STAINS AND REAGENTS

(IN COLLABORATION WITH F. HAYNES)

1. Chief Solvents for Stains—2. Hæmatoxylin Stains—3. Carmine Stains—4. Various Aniline Dyes—5. Special Stains and Reagents —6. Stains especially used in Pathology and Bacteriology— 7. Mounting and Ringing Media—8. Mixture for Cleaning Slides and Cover-slips.

A SPECIAL bench, kept apart for staining, is desirable. There should be at least one sink. It is useful to have a couple of lengths of glass rod stretching across the sink, about $1\frac{3}{4}$ inches apart, fastened to the bench at the ends by plasticine, in order that, when using stains from drop-bottles, slides may be placed on this rack.

The rest of the permanent furniture of the bench should comprise a Bunsen burner and a hot plate for the hardening of the balsam of mounted slides. The hot plate should be kept at a temperature of 45° to 50° C.

Wherever other considerations (see below) allow, reagents should be kept in ground-glass-stoppered slide tubes (see Fig. 14, B), owing to the economy both of time and material which these effect over drop-bottles. Frequently used stains should always be kept in tubes, unless indeed the custom is to stain many slides simultaneously, when slide dishes which will hold six to twenty slides (see Fig. 14, C) should be used. Cheaper but less convenient than the glass-stoppered slide tube is the variety provided with a cork (Fig. 14, A).

Tubes should always be used for xylol and the various strengths of alcohol used for clearing and dehydration, since the time occupied by these processes can be materially reduced

by moving the slide about in the reagent by means of a blunt-ended forceps.

The advantages of using tubes are that economy of time and material is effected, since at least three slides can be immersed at the same time in the reagent, which can be used many times over. Furthermore, many of the common reagents improve on use to a certain degree—*e.g.* hæmatoxylin in iron-hæmatoxylin staining.

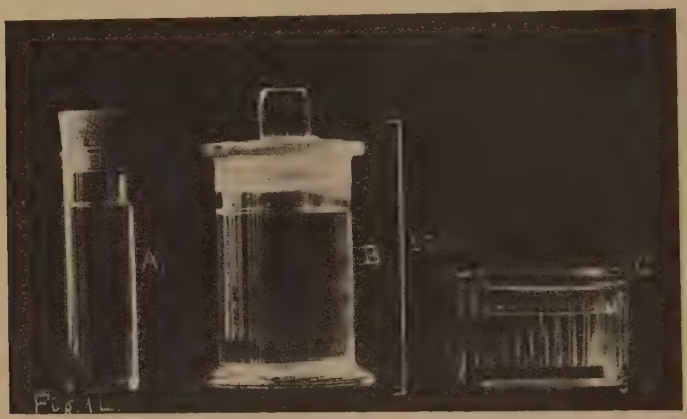


Fig. 14.—Vessels used for Staining and otherwise treating Sections on the Slide.

A. *Slide Tube.* Only accommodates one or two slides (if placed back to back). Cheaper than, but inferior to :

B. *Slide Jar.* The most useful form of vessel. A number of slides may be arranged around the periphery of this.

C. *Slide Dish.* Suitable for staining a number of slides. By placing these back to back two can be accommodated in each groove, making a total of twenty to twenty-four slides. *Not* suitable for volatile substances (*e.g.* alcohol, benzol, or solutions of dyes in high-grade alcohol), as the cover does not fit sufficiently well.

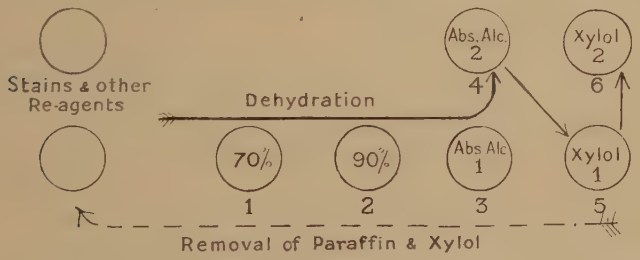
On the other hand, the active ingredients in some reagents become exhausted by use, *e.g.* sodium thiosulphate for the removal of iodine, and tap-water substitute, therefore the tubes of these substances should be frequently replenished. Also, the slide on being placed in a tube of reagent always carries with it a certain amount of those reagents with which it has been previously treated. If the earlier reagents are soluble in the later, this will eventually become impure.

Naturally, the contents of the tubes must be changed after a reasonable amount of use, which is always indicated by the abnormal reaction of the reagent.

The following substances may advantageously be kept in slide tubes (slide dishes may be used in all cases save those of volatile substances, which evaporate too readily when kept in dishes) :

Xylol ; the various grades of alcohol ; 0·4 per cent. iodine in 70 per cent. alcohol ; sodium thiosulphate solution ; hæmatoxylin (of Ehrlich or Delafield) ; hæmatoxylin (Heidenhain) ; iron alum ; tap-water substitute ; eosin ; van Gieson ; Weigert's elastin stain ; methylene-blue ; hydrochloric acid alcohol ; lithium carbonate alcohol ; safranin ; lichtgrün ; Mallory's connective - tissue stain ingredients ; carmine ; orange G, etc. etc.

The tubes containing the solutions necessary for any one staining method should be kept in a row together, in the order of their progressive use, as should also the reagents used in dehydrating and clearing, thus :



In dehydrating, the slide should go in order from 1 to 6, passing through absolute alcohol 1 to absolute alcohol 2, then to xylol 1, and finally to xylol 2 (see continuous arrow line). In removing the paraffin from a section in order to bring it down to water, the order would be 5, 3, 2, 1, Nos. 4 and 6 being thus kept more pure for the dehydrating and clearing. (See dotted arrow line.)

Drop-bottles should only be employed for stains in infrequent use, and for the Romanowsky stains (Leishman, Giemsa, Wright, etc.) ; also for stains which require to be steamed on the slide. A wash-bottle capable of delivering a rapid stream of water should be kept filled with distilled water

for washing slides after the use of stains which are sensitive to traces of alkali (van Gieson, Leishman).

One of the most important articles of furniture of the staining bench is a microscope for use in controlling the degree of staining and differentiation. This should carry a 16 mm. ($\frac{2}{3}$ inch) and an 8 mm. ($\frac{1}{6}$ inch) objective and a No. 2 eye-piece.

Cover-slips of two or three sizes should be kept, each size in a separate Petri dish, ready cleaned for use. If obtained from a reputable firm they should require no cleaning further than that of being kept in hydrochloric acid alcohol until a batch is dried carefully on a grease- and lint-free duster and placed in the Petri dishes. If they are very greasy, treat with potassium bichromate and sulphuric acid mixture (p. 120) for a few hours, rinse thoroughly first in running tap water, then in distilled water. Finally, dip in alcohol. Then change your dealer. These remarks apply also to slides.

1. CHIEF SOLVENTS FOR STAINS

The commonest solvents are :

Water.—Should always be distilled unless otherwise stated.

Alcohol.—Ethyl alcohol of various concentrations.

Methyl alcohol—usually absolute. Used especially in the preparation of blood stains, for which purpose it must be *acetone free.*

Aniline Water.—Add 10 c.c. of aniline to a half or 1 litre of hot distilled water in a flask. The mixture is well shaken and cooled. Then filter into a stock bottle.

Phenol.—Employed in aqueous solutions of 0·5 to 5 per cent.

Acetone.—Generally used in combination with alcohol in certain fat stains.

2. HÆMATOXYLIN STAINS

Delafield's Hæmatoxylin

Prepare 400 c.c. of a saturated solution of ammonia alum (this is about 1 part of alum to 11 of distilled water).

Dissolve 4 grammes of hæmatoxylin in 25 c.c. of absolute

alcohol. Mix. Leave exposed to light in a flask with a cotton-wool plug for three to four days. Filter.

Add to the filtrate 100 c.c. of glycerin and 100 c.c. of methylic alcohol.

Ripen in a warm place for six weeks at least. It is well to shake the bottle vigorously once daily to accelerate the oxidation. The ripe solution will keep for years in a well-stoppered bottle.

NOTE.—In this, as in Ehrlich's hæmatoxylin, the mordant is combined with the staining solution. The reason why "ripening" is necessary is because hæmatoxylin itself is a very diffuse stain, quite useless for staining. During the ripening process it becomes partially converted into a higher oxidation product, which is hæmatein. Light, warmth, and exposure to the oxygen of the air greatly accelerate this conversion.

Ehrlich's Acid Hæmatoxylin

Hæmatoxylin	2 grms.
Absolute alcohol	100 c.c.
Glycerine	100 c.c.
Distilled water	100 c.c.
Glacial acetic acid	10 c.c.
Potash alum in excess (Formula: $K_2SO_4.Al_2(SO_4)_3.24H_2O$).	

Dissolve the hæmatoxylin in the alcohol before adding the other components. Ripen as for Delafield's hæmatoxylin. When sufficiently ripe, the stain has considerably darkened and looks exactly like good port wine. Then store in a well-stoppered bottle, in which the stain keeps without deterioration for many months.

Heidenhain's Iron-Hæmatoxylin

A. THE ALUM BATH (USED AS A MORDANT AND DIFFERENTIATOR)

Iron and ammonia alum ($(NH_4)_2.Fe_2(SO_4)_4.24H_2O$)	5 grms.
Distilled water	100 grms.

For differentiation (especially by the beginner) the solution should be diluted to 2 to 3 per cent.

B. The Hæmatoxylin Bath

Hæmatoxylin	0·5 grm.
96 per cent. or absolute alcohol	10 c.c.
Distilled water	90 c.c.

A good brand of hæmatoxylin is essential. Dissolve it first in the alcohol; then add the water. The solution takes a few days to ripen, *i.e.* for the hæmatoxylin to undergo partial oxidation into hæmatein. I find that the above solution keeps very well in a stoppered bottle. Keep a stock solution of this, on account of the time needed for ripening. You can use hæmatein in place of hæmatoxylin, in which case the solution stains with intensity as soon as prepared. But there appears to be a danger of the hæmatein becoming converted into higher oxidation products—which do not stain—on keeping. The hæmatoxylin (or hæmatein) solution must be kept in well-stoppered bottles or slide jars, to avoid the appearance of moulds.

MAYER'S HÆMALUM

Hæmatein	1 grm.
90 per cent. alcohol	50 c.c.
Potash alum	50 grms.
Distilled water	1000 c.c.

Carefully dissolve the hæmatein with moderate warmth in the alcohol. Then add this to the solution of alum. Cool and filter. The solution keeps fairly well.

The addition of 20 c.c. of glacial acetic acid to the above makes "Mayer's acid hæmalum"—a very powerful *nuclear* stain.

3. CARMINE STAINS

Best's Carmine Stain for Glycogen

CARMINE SOLUTION

Carmine	2 grms.
Potassium carbonate	1 grm.
Potassium chloride	5 grms.
Distilled water	60 c.c.

Carefully warm this for a few minutes. Cool and add 20 c.c. of concentrated ammonia. This solution must be kept in a tightly stoppered bottle in a dark, cool place.

It keeps for several weeks in summer, and two months (or longer) in winter. Filter before use.

THE DIFFERENTIATOR

Absolute methyl alcohol	40 c.c.
Absolute ethyl alcohol	80 c.c.
Distilled water	100 c.c.

Mayer's Mucicarmine

Carmine rubr. opt.	1·0 grm.
Pure aluminium chloride	0·5 grm.
Aq. dest.	2·0 c.c.

Heat the above over a flame in a small porcelain capsule for about two minutes. Then make up to 100 c.c. with 50 per cent. alcohol. Allow to stand for twenty-four hours. Then filter.

Before use dilute one volume of stain with five to ten volumes of tap water (the more alkaline, the better).

Mayer's Paracarmine

Carminic acid	1·0 grm.
Aluminium chloride	0·5 grm.
Calcium chloride	4·0 grms.
70 per cent. alcohol	100·0 c.c.

When dissolved, filter. The solution keeps indefinitely.

Mayer's Hydrochloric Acid Carmine

Carmine	4 grms.
Distilled water	15 c.c.
Concentrated hydrochloric acid	30 drops.

Boil to dissolve the carmine. Then add 95 c.c. of 85 per cent. alcohol and filter while hot. Finally neutralise by adding ammonia till the carmine just begins to precipitate. Filter again when cold. The solution keeps well.

4. SOLUTIONS OF VARIOUS ANILINE DYES

Altmann's Acid Fuchsin

Aniline water (see p. 106) . . . . 100 c.c.
Acid fuchsin to saturation (*i.e.* 5 to 7 grms.).

NOTE.—According to the original formula a 20 per cent. solution of acid fuchsin should be used. As pointed out by Gatenby, the solubility of acid fuchsin is far less—being rather more than 5 per cent.

Methylene-Blue

Borrel's method of ripening (*i.e.* partially oxidising) methylene-blue gives a very useful solution for most purposes. Langeron's method of preparing it gives very good results.

BORREL'S METHYLENE-BLUE

A. Make a 1 per cent. solution of methylene-blue (chemically pure) in distilled water by heating on the water bath.

B. Dissolve 0·5 gramme of silver nitrate in 100 c.c. of distilled water in a perfectly clean flask of about 500 c.c. capacity.

Add gradually to *B* a 3 per cent. solution of sodium or potassium hydrate. Shake the flask after each addition of the alkali. A brown precipitate of silver oxide is formed. Stop adding the alkali as soon as no more precipitate comes down. Allow the latter to settle, decant the supernatant fluid, and wash the precipitate by shaking it with distilled water.

Repeat the process until the distilled water is no longer alkaline to litmus paper. Pour off the distilled water.

Add solution *A* to the silver oxide. Boil gently for five minutes and allow to cool. The solution should be dark violet when cold. If still blue, boil it again to oxidise the methylene-blue still further. If it be distinctly reddish, add a little 1 per cent. methylene-blue, until violet. Usually the solution is of the correct colour if prepared as above.

Filter and store in a stoppered bottle. The solution keeps well.

For use, dilute with distilled water in the proportion of 1 : 5 of distilled water.

NOTES.—The flasks, etc., must be perfectly clean and washed with distilled water. Pure reagents are essential. Suitable varieties of methylene-blue are "methylene-blue: medicinal," or "Methylene-Blue rectif. nach Ehrlich" of Grübler.

Eosin

For ordinary purposes use a good brand of water-soluble eosin such as eosin G, eosin A.G, or eosin 3G.

The most useful solution is a 5 per cent. aqueous solution.

A 1 per cent. solution can always be made from this. A mould is particularly liable to infect eosin solutions. Its growth may be partly inhibited by the addition of three to four drops of formol to every 100 c.c. of the stain. Filtration of an infected solution makes it as good as new.

Mann's Methyl-Blue Eosin

1 per cent. aqueous solution methyl-blue	35 c.c.
1 per cent. aqueous solution eosin	45 c.c.
Distilled water	100 c.c.

The solution keeps indefinitely.

Methyl-Green Acetic

A saturated solution of methyl-green in 0·5 per cent. or 1 per cent. acetic acid. It keeps fairly well.

Erythrosin

Masson's formula for this is :

Erythrosin	1 grm.
Distilled water	100 c.c.
40 per cent. formol	4 to 5 drops.

(The formol is added to inhibit the growth of moulds.)

As with eosin, moulds are apt to form in course of time. But the solution is not injured and need only be filtered.

Masson's Saffron [1]

Saffron is the dried stigmata of *Crocus sativus*.

Place in a flask 2 grammes of saffron and 100 c.c. of distilled water. Boil gently for an hour in a water bath. Filter. Then add to the filtrate 1 c.c. of 40 per cent. formol and 1 c.c.

[1] Although included here for convenience sake, saffron is not an aniline dye.

of a 5 per cent. aqueous solution of tannic acid. This solution should be kept in a dark glass bottle. It may deteriorate after several weeks. The formol is added to inhibit the growth of moulds.

Orange G

Either a saturated solution in absolute alcohol or a 0·5 per cent. solution in distilled water. Both solutions keep well.

Lichtgrün

Also known as Vert Lumière and Light Green.

A saturated solution in 90 per cent. alcohol. The solution keeps indefinitely.

Safranin

Safranin	1 grm.
Aniline water (see p. 106) . .	48 c.c.
96 per cent. alcohol . . .	52 c.c.

This formula gives rapid and precise staining. A good sample of safranin (*e.g.* safranin O of Grübler) is essential.

An alternative is a 1 per cent. aqueous solution of the dye. These solutions keep indefinitely.

Unna's Pyronin-Methyl-Green

Methyl-green (pure)	0·15 grm.
Pyronin	0·25 grm.
96 per cent. alcohol	2·5 grms.
Glycerine	20 c.c.
0·5 per cent. aqueous solution of phenol to	100 c.c.

The solution keeps indefinitely.

Van Gieson's Stain

An excellent combination, recommended by Schmorl, is :

Concentrated aqueous solution of picric acid .	150 c.c.
Concentrated aqueous solution of acid fuchsin .	3 c.c.

The solution keeps well.

Curtis's Ponceau S Substitute for van Gieson

Non-fading—in contrast to van Gieson's stain—but not so precise for young collagen tissue.

1 per cent. aqueous solution Ponceau S (Grübler) .	10 c.c.
Saturated aqueous solution of picric acid . .	90 c.c.
2 per cent. glacial acetic acid . . .	1 to 2 c.c.

The solution keeps well.

Weigert's Elastin Stain

The nature of this stain is unknown beyond that it contains organically combined iron. The stain is presumably a compound formed by the combination of the basic fuchsin molecule with that of the iron compound of the phenol.

In Weigert's original method the use of resorcin is recommended, though he states that any other phenol may be used. We have had extensive experience of both resorcin-fuchsin and phenol-fuchsin, and find the latter better.

The advantages of phenol-fuchsin are :

1. It is far cheaper than resorcin-fuchsin.
2. The solution keeps better. Resorcin-fuchsin deteriorates after six weeks or so, whereas phenol-fuchsin solutions over a year old are still efficacious.
3. Staining with phenol-fuchsin is quite as specific as with resorcin-fuchsin, with the advantage of a darker staining of elastic fibres.

We have found the following modification of Weigert's original method to give excellent results :

(1) Dissolve in 100 c.c. of distilled water 1 gramme of basic fuchsin and 2 grammes of phenol. Raise to boiling-point in a porcelain or glass vessel of at least 250 c.c. capacity.

(2) When boiling, gradually add 12·5 c.c. of a 30 per cent. solution of ferric chloride ($FeCl_3$). (This corresponds to the *Liquor ferri sesquichlorati* P.G. of Weigert.) A precipitate is formed. Continue the boiling, with stirring, for at least five minutes, to render the precipitate more granular.

(3) Cool and filter, using a Buchner filter flask and water

suction pump. Wash the precipitate on the filter with distilled water until the runnings are practically colourless.

(4) Dry the precipitate over a water bath if not required for immediate use. It is recommended that ten times the above quantities be taken, and the dried precipitate (which keeps indefinitely) be kept for making up as required. To make the staining solution :

(5) Boil, under a reflux condenser, 0·75 gramme of the dry precipitate with 100 c.c. of 96 per cent. alcohol for twenty minutes. Cool, filter, and add 2 c.c. of concentrated hydrochloric acid. If you have no reflux condenser, plug a flask with cotton wool and warm the alcohol on a water bath.

5. SPECIAL STAINS AND REAGENTS

Cajal's Method for the Golgi Apparatus

A. THE FIXATIVE

Uranium nitrate	1 grm.
40 per cent. formol	15 c.c.
Distilled water	85 c.c.

For the central nervous system or for tissues difficult to impregnate, fix in the following fluid :

Uranium nitrate	1 grm.
Absolute ethyl or methyl-alcohol	30 c.c.
40 per cent. formol	15 to 20 c.c.
Distilled water	80 c.c.

Da Fano's modification :

Cobalt nitrate	1 grm.
40 per cent. formol	15 c.c.
Distilled water	100 c.c.

These fluids keep indefinitely.

B. THE REDUCING FLUID

Hydroquinone	2 grms.
40 per cent. formol	15 c.c.
Distilled water	100 c.c.
Sodium sulphite (Na_2SO_3)	0·5 grm.

This solution darkens with age, but is still efficacious though, possibly, its reducing powers are somewhat slowed down.

C. THE TONING SOLUTIONS

Sodium thiosulphate . . .	3 grms.	Solution 1.
Ammonium sulphocyanide . .	3 grms.	
Distilled water	100 c.c.	
Gold chloride	1 grm.	Solution 2.
Distilled water	100 c.c.	

Keep in separate bottles. Mix in equal parts before use. The separate solutions keep quite well.

Leishman's Stain

The stain is best bought in solution or in powder. It must be procured from a reliable firm (*e.g.* Gurr or Grübler). To make a solution you will need absolute methyl-alcohol, *acetone-free.* The last reservation is particularly important, and should be specified when ordering methyl-alcohol for making up this or other blood stains. If the methyl-alcohol contains a trace of acetone, the stain may work at first, but will soon deteriorate. A sample of methyl-alcohol in which you can smell acetone is useless.

Dissolve the stain as follows :

Leishman's stain, in powder . . .	0·15 grm.
Pure absolute methyl-alcohol . . .	100·0 c.c.

Have ready the 100 c.c. of methyl-alcohol, a small pestle and mortar, and a dry bottle with a ground-glass stopper (or a large drop-bottle). Grind up a little of the powder in the mortar with some of the methyl-alcohol ; then pour it into the bottle. Repeat this until you have used all the powder and alcohol. The solution is best left to stand overnight before using it, so as to ensure complete solution.

The Leishman's solution for the staining bench must be kept in a drop-bottle. After use, always turn the stopper so as to keep out air from the solution.

Leishman's stain keeps for months, provided it be in an airtight vessel.

Giemsa's Stain

This is best bought from a reliable source (Gurr or Grübler) ready made up in a concentrated solution, which is diluted immediately before use. If the solution be stored in a well-stoppered bottle it will keep for months, if not years.

If you wish to dissolve the ingredients yourself, you take them as follows :

Azur II.—eosin	3·0 grms.
Azur II.	0·8 grm.
Pure glycerine	125·0 grms.
Pure methyl-alcohol (*acetone-free*)	375·0 grms.

Store the solution in an absolutely clean glass-stoppered bottle. But it is far better to buy it ready made up.

Wright's Blood Stain

Either buy this in solution or dissolve the powder in pure (acetone-free) methyl-alcohol. It is a waste of time to prepare the stain in the laboratory. The general procedure for making up the solution is as for Leishman's stain (p. 115). The proportions are :

Wright's blood stain, in powder	0·15 grm.
Pure methyl-alcohol	100·0 c.c.

6. STAINS ESPECIALLY USED IN PATHOLOGY AND BACTERIOLOGY

Aniline Water—Gentian Violet

Aniline water (see p. 106)	100 c.c.
Saturated solution of gentian violet in 96 per cent. or absolute alcohol	11 c.c.

The solution keeps well.

Carbol-Fuchsin

Basic fuchsin	1 grm.
Absolute alcohol	10 c.c.
5 per cent. phenol in distilled water	100 c.c.

Dissolve the basic fuchsin in the alcohol, then mix with the phenol solution.. The stain keeps indefinitely.

Iodine Solutions

Of Gram.		*Of Lugol.*
Iodine	1 grm.	1 grm.
Potassium iodide	2 grms.	2 grms.
Distilled water	300 c.c.	100 c.c.

These solutions lose colour and their effect weakens after being kept for several months. This is particularly the case if the bottle be nearly empty.

Manson's Methylene-Blue

Methylene-blue (pure medicinal)	2 grms.
Borax	5 grms.
Distilled water	100 c.c.

The solution improves—through partial oxidation into methylene-azur—on keeping.

Nicolle's Carbol Thionin

2 per cent. aqueous solution of phenol	4 parts.
Thionin, saturated solution in 60 per cent. alcohol	1 part.

An easier solution to prepare is Besson's modification :

Thionin	1 grm.
Phenol, cryst.	1 grm.
80 per cent. alcohol	10 c.c.
Distilled water	100 c.c.

Carbol thionin keeps fairly well.

7. MOUNTING AND RINGING MEDIA

Xylol-Dammar

REFRACTIVE INDEX, 1·52

Preparation.—Dissolve dammar in xylol to make a fairly thin solution. This may be done in the incubator or paraffin thermostat.

Then filter through a large filter paper. If the solution be still too thin for mounting purposes, place it uncovered in the paraffin oven or incubator until of a syrupy consistency.

Keep the bench solution in a balsam bottle. When it goes acid to litmus, throw it away. The stock solution is best kept in the dark.

Dammar—in contrast to balsam—does not go brown on keeping, and is hence preferable.

Xylol-Balsam

REFRACTIVE INDEX, 1·524

Prepare exactly as for dammar.

Other Varieties of Dammar and Balsam

E.g. chloroform and origanum balsam—are prepared in the same way.

Dilute Glycerine

REFRACTIVE INDEX, 1·397

Pure glycerine and distilled water, equal parts.

Cedar Oil and Clove Oil

REFRACTIVE INDICES, 1·52 and 1·533

are sometimes used as temporary mounting media. With the former, an oil immersion can be used without a cover-slip.

Euparal

REFRACTIVE INDEX, 1·483

A mixture of eucalyptol, sandarac, camsal, and paraldehyde. Buy the colourless variety (there exists a green euparal which contains a copper salt) and keep it in a balsam bottle.

You can mount either from xylol or direct from alcohol of 80 per cent. and upwards. This medium is useful for rapid

work and also, as pointed out by Bolles Lee, for use when a medium of a lower refractive index than dammar or balsam is required. For clearly seeing certain cytoplasmic structures, such as unstained spindle fibres, this medium is strongly recommended by Lee.

Gurr's Neutral Mounting Medium

REFRACTIVE INDEX, 1·51

A useful preparation in which blood stains such as Leishman or Giemsa may be mounted without fading. Buy it from the maker. Miscible in alcohol of 90 per cent. or over. Not miscible with xylol. The best way to use this is to dip the slide in 96 per cent. or absolute alcohol, blot it, then mount at once in the medium. For blood films, blot and dry from aq. dest. and mount direct.

Glycerine Jelly

REFRACTIVE INDEX, *circa* 1·47

Gelatine	10 grms.
Distilled water	60 c.c.
Pure glycerine	70 c.c.
Phenol cryst.	0·25 grm.

Dissolve the gelatine in the water in a beaker placed in a water bath. When the gelatine has dissolved, add the glycerine, and, finally, the phenol. Keep this medium either in a corked tube or in a thin-walled balsam bottle. To melt it, place the vessel in hot water.

Apathy's Mounting Medium

REFRACTIVE INDEX, 1·524

Pure gum arabic	50 grms.
Pure cane sugar	50 grms.
Distilled water	50 grms.
Thymol	0·05 grm.

Keep in a well-stoppered bottle. This medium sets quite hard.

Lactophenol of Amann

REFRACTIVE INDEX, 1·44

Pure phenol cryst.	10 grms.
Lactic acid	10 grms.
Glycerine	20 grms.
Distilled water	10 grms.

Exposure to light causes this medium to go yellow. This is not of any importance, but can be largely avoided by keeping lactophenol in the dark, or in a brown glass balsam bottle. A fluid medium, needing to be ringed for permanent preparations.

RINGING MEDIA

For fluid and semifluid mounts.

Ordinary paraffin.—Applied to the border of the cover-slip with a hot L-shaped wire, may be used.

Krönig's Deckglaskitt is the best ringing medium that I am acquainted with. It is supplied, with a useful instrument for applying it, by Leitz. To use it, hold the metal end of the special instrument in a flame for a few seconds, press it into the compound in the box, then apply it to the junction of cover-slip and slide. This medium sets extremely hard.

To make it up, add gradually 7 to 9 parts of colophonium resin to 2 parts of melted wax. When thoroughly mixed, filter and cool.

8. MIXTURE FOR CLEANING SLIDES AND COVER-SLIPS

Concentrated sulphuric *or* nitric acid.	1 part.
2 to 3 per cent. potassium bichromate	9 parts.

Soak in this, wash in running, then in distilled water. Either store in acid alcohol or dip each slide (or cover-slip) into 90 or 96 per cent. alcohol and dry with an absolutely clean cloth.

Avoid getting the cleansing fluid on the hands.

CHAPTER IX

TYPE STAINING METHODS

A. Staining of Paraffin Sections: Outline of Procedure—Additional Treatment for Tissues fixed in Mercuric Chloride.

"*Type*" *Micro-Anatomical Methods*: 1. Delafield's Hæmatoxylin and Eosin—2. Ehrlich's Hæmatoxylin and Eosin—3. Hæmatoxylin (of Delafield or Ehrlich) and van Gieson—4. Safranin and Weigert's Elastin Stain—5. Weigert's Elastin and van Gieson—6. Mallory's Connective-Tissue Stain—7. Eosin and Methylene-Blue—8. Mann's Methyl-Blue-Eosin—9. Masson's Hæmatoxylin-Erythrosin-Saffron.

"*Type*" *Cytological Methods*: 1. Heidenhain's Iron-Hæmatoxylin—2. Heidenhain's Iron-Hæmatoxylin in combination with other Stains (Eosin; Orange G; van Gieson; Lichtgrün)—3. Champy-Kull Technique—4. Cajal Technique for the Golgi Elements, and Da Fano's Modification—5. Pyronin-Methyl-Green.

B. Staining Celloidin Sections.

C. Staining Frozen Sections.

OUTLINE OF PROCEDURE FOR STAINING PARAFFIN SECTIONS

Paraffin sections are always stained on the slide after attachment thereto by the method already described (see p. 78).

Before staining, certain preparatory treatment of the sections is necessary, and this involves the following steps. *The reagents mentioned in these paragraphs may be either poured from drop-bottles over the sections (in which case the slide should be flooded at least once, tilted, and flooded again), or the slides placed in slide tubes or dishes of the solutions. This is far the best method.*

1. Removal of the Paraffin Wax from the Sections

Procedure.	Raison d'être.
Treat with xylol for one to three minutes.	The tissues in the section are impregnated with paraffin wax and consequently nearly impermeable to stains and other reagents. It is therefore imperative to remove all traces of paraffin wax.
It is advantageous to warm the slide (section upwards) above a Bunsen burner until the paraffin is *just* melted before treating with xylol.	The rate of solubility of the paraffin in the xylol is enormously increased. By moving the slide (after warming) in a large staining jar of xylol all the paraffin may be removed in thirty seconds from a section of average thickness (*i.e.* not more than 10 μ).

2. Removal of the Xylol by Absolute Alcohol

Treat with absolute alcohol for one-half to one minute.	Xylol is practically insoluble in aqueous media or in low-grade alcohol. Whence the necessity for removing all traces of it before the sections can be stained or otherwise treated.

3. Treatment with Descending Grades of Alcohol

(*a*) Treat with 90 per cent. alcohol. Half a minute suffices provided the slide be moved about in the medium. (*b*) Treat with 70 per cent. alcohol as for 90 per cent. alcohol.	The reduction of diffusion currents (such as are caused if the sections are taken from absolute alcohol direct into water), which sometimes cause them to become detached. *For rapid work* the lower (70 per cent.) grade can be omitted. *For extremely delicate work* (*e.g.* films of Protozoa) pass through 50 per cent. and 30 per cent. alcohol.

4. Water

Place the slide in a slide dish or tube of tap water. For certain special stains, distilled water is used for rinsing sections.

Most stains—*e.g.* Delafield's hæmatoxylin—are made up in aqueous solutions. Consequently the minimum of contamination of the stain and the minimum of diffusion disturbances are caused by passing from water to the staining medium.

NOTE.—In the case of stains dissolved in alcohol, proceed direct from the nearest grade of alcohol to the stain.

5. Staining the Sections

This may comprise (*a*) treatment with a single stain; (*b*) successive treatment by two stains, of which the second is known as the "Counterstain," while the whole process is termed "double staining"; (*c*) the successive application of three stains—"Triple staining"; (*d*) the application at one and the same time of a mixture of two or three stains.

The technique of staining cannot be further dealt with in general outline, owing to the individuality of the technique for each stain.

Progressive stains do not require differentiation; regressive stains do. But progressive stains usually yield better results if the material is first overstained, then differentiated.

6. Dehydration of the Sections

Treat with ascending grades of alcohol in the reversed sequence in which you employed them preparatory to staining—*e.g.* 70, 80, and 100 per cent.

The time for which the slide is left in each grade of alcohol is dependent on the stain employed.

Very roughly speaking, it may be said to be about one minute in the grades up to absolute alcohol, and one to two minutes in the latter.

Before the sections can be cleared all traces of water must be removed, since this substance is immiscible with most clearing media, *e.g.* xylol, the "type" clearing agent.

Certain regressive stains are differentiated in alcohol; in such instances it is the aim of the histologist to achieve both the right degree of extraction of the stain and proper dehydration of the stain.

7. Clearing

Treat with xylol—one minute or more. Most stains are insoluble in this medium, and sections can therefore be left in it as long as desired. Note, however, that eosin, orange G, and certain other aniline dyes are very slightly soluble in xylol. This means that a sojourn of several days in xylol preparatory to mounting extracts a good deal of the dye.

Certain stains are soluble in alcohol. Consequently any traces of this must be removed if such stains are not to fade when mounted. Xylol, furthermore, clears the section, *i.e.* it raises its refractive index—whereby causing greater transparency—to a point akin to that of the mounting medium.

8. Mounting

The standard medium is a solution of Canada balsam or dammar in xylol. Although fluid at first it soon sets—especially under the influence of heat.

Once the section is mounted it is encased in a medium of a suitable refractive index for microscopic observation and one, furthermore, in which it can be permanently preserved.

The section is now ready for microscopic observation.

ADDITIONAL TREATMENT REQUIRED FOR TISSUES FIXED IN MERCURIC CHLORIDE

This is essential for all tissues which have been fixed in mercuric chloride or in any fixative containing this salt.

Exception: very small fragments of tissue which have received iodine treatment during dehydration previous to being sectioned (see p. 36). Larger pieces of tissue are best treated in a similar manner with iodine-alcohol followed by a repetition of the process when the sections are on the slide. The idea of this is to remove the gross $HgCl_2$ deposit before sectioning (in order to save the microtome knife) while the finer precipitate is dealt with later and more effectively in the section-on-the-slide stage.

The exact nature of the chemical combination which occurs during removal of $HgCl_2$ from tissues by treatment with iodine is not definitely known.

PROCEDURE.—Go through stages 1 to 3 (*a*) of general scheme.

Transfer the sections to a 0·5 per cent. solution of iodine in 70 per cent. alcohol. Leave therein for a half to two minutes. Rinse in water. Remove the yellow to brown iodine stain from the sections by treatment with the following solution of sodium thiosulphate (" Hypo ") until the natural colour of the sections has reappeared—usually a half to two minutes.

Sodium thiosulphate ($Na_2S_2O_3$) .	7·5 grms.
96 per cent. alcohol . . .	100·0 c.c.
Aq. dest.	900·0 c.c.

Add a crystal of thymol to the stock bottle.

The displacement of the iodine is due to the following combination :

$$2\,Na_2S_2O_3 + I_2 = 2NaI + Na_2S_4O_6.$$

Rinse in water ; stage 4 of the general scheme ; after which you stain, dehydrate, and clear in the ordinary manner.

The entire process, in relation to the general scheme, may be summarised as follows :

Xylol.
↓
Absolute alcohol.
↓
90 per cent. alcohol.
↓
Iodine in 70 per cent. alcohol.
↓
Water.
↓
" Hypo " (*i.e.* treatment with $Na_2S_2O_3$).
↓
Water.
↓
Staining, dehydration, clearing, etc., as usual.

NOTES.—(i) If you do not know whether the tissue has been fixed in $HgCl_2$ or not, examine the sections with the staining microscope at stage 3 (*a*) (see p. 122). $HgCl_2$ precipitate, if present, will appear as a fine, brown, granular deposit, usually less abundant at the periphery than in the centre of the section, owing to the solubility of the salt in alcohol during dehydration.

(ii) In contrast with endogenous pigments the granules may be seen with the 4 mm. ($\frac{1}{6}$ inch) objective to lie on *top* of the cells and tissues.

(iii) Treatment of tissues—*e.g.* by error—*not* fixed in $HgCl_2$ with I_2 and $Na_2S_2O_3$ does them no harm. It is merely a waste of time.

"TYPE" MICRO-ANATOMICAL STAINS

Advice to the Beginner.—Staining is an art which can only be acquired by individual practice. The best way to attain proficiency is to show one's slides to a competent critic, failing that to compare one's own finished specimens with really good microscopic preparations, and, above all, not to rest contented until a genuine standard of perfection has been attained. With practice, staining methods become practically reflex.

1. Delafield's Hæmatoxylin and Eosin

Take the sections down to water (steps 1 to 4 of the general scheme, plus iodine treatment, if necessary).

(1) Transfer to hæmatoxylin. Stain two to ten minutes. If the solution be a " ripe " one, the former time (or even less) will suffice ; if only recently prepared, a longer staining period is indicated.

(2) Rinse in water.

(3) Blue the sections *either* in a slide dish through which a gentle stream of tap water has been caused to run for two to ten minutes (the time being dependent on the alkalinity of the water), *or* place them in *Scott's Tap-Water Substitute*, which will blue them in one-quarter to one minute. This is the best procedure.

Sodium bicarbonate . . .	3·5 grms.
Magnesium sulphate . . .	20·0 ,,
Tap water	1000·0 c.c.

Add a crystal of thymol to the stock solution.

Dissolve the salts separately. Then mix.

(4) Examine with the staining microscope. The cell nuclei should be a bright and transparent blue, the cytoplasm—with the exception of mucin and the basophil granules of certain cells (*e.g.* mast cells)—colourless. *If insufficiently stained* : rinse in water and repeat the staining and bluing processes. *If overstained* : dip the slide into a staining jar of weak HCl (0·5 per cent. aq. soln.), leave therein for five to

thirty seconds, rinse promptly in water, blue the sections again and re-examine with the microscope. If still overstained, redifferentiate in weak HCl.

(5) Transfer the sections to water.

(6) Counterstain in a 5 per cent. aqueous solution of eosin. Usually one-quarter to one minute suffices.

(7) Rinse rapidly in water.

(8) Examine rapidly with the staining microscope to control the depth of the counterstain. The cytoplasm of the cells should be a bright transparent pink at this stage ; so should collagen connective-tissue fibres, red blood corpuscles, etc.

(9) Dehydrate and clear (stages 6 to 8 of the general scheme) bearing the following point in mind :

Eosin (including the water-soluble forms) is rapidly taken out of tissues by 70 per cent. and 90 per cent. alcohol, more slowly by absolute alcohol. *Therefore you must allow for this by slightly overstaining* the sections and rapidly dehydrating them in alcohol (*i.e.* half minute in 70 per cent. and 90 per cent. grades, one minute in absolute alcohol).

If the sections are much overstained with eosin, remove it by leaving them for as long as necessary in 70 per cent. alcohol. Another excellent differentiating medium for eosin is tap-water substitute, which being alkaline differentiates eosin—an acid dye—quite rapidly.

(10) Mount in dammar or balsam.

Nuclei, blue ; karyosomes, dark blue ; plasmosomes, red ; cytoplasm — except when basophil—in shades of pink ; muscle and collagen fibres, pink, also elastic fibres (when thick) ; red blood corpuscles, a bright pink, likewise the thyroid colloid and keratin. The preparation should have a light and transparent aspect under the low power.

Specialised structures such as neuroglial fibres, dendrites, etc., are not brought into evidence. Nor are the majority of the cell components —*e.g.* the Golgi bodies, glycogen, or mitochondria, even when not destroyed by the fixative. Occasionally the latter, after appropriate fixation, may be seen faintly indicated. An example of this is to be seen in the ordinary (exocrine) cells of the pancreas, the basal poles of which often show a vague blue striation. This appearance is due to the rod-like mitochondria, concentrated in this region of the cells, being diffusely stained.

The eosin stain is permanent, but the hæmatoxylin usually goes red in course of time. This is due to the balsam becoming acid. It occurs

especially at the edges of the cover-slip. Sections mounted in balsam (neutral), suitably dried on the hot plate, and then kept out of the sun, keep for many years.

NOTES.—(1) *Progressive* v. *Regressive Methods.*—Delafield's hæmatoxylin can be used either as a progressive or a regressive stain. In the latter case the section is grossly overstained, then differentiated with weak HCl to the right degree of nuclear sharpness. Although the best results are obtained by the regressive method, pictures nearly—and sometimes quite—as clear are obtained by the progressive technique, which is quicker and suffices for routine purposes.

If your hæmatoxylin has been recently made up, your only hope is grossly to overstain (ten to fifteen minutes or even longer), then to differentiate.

(2) *Time required in the Hæmatoxylin.*—The time tissues require to be stained is not only a function of the degree of ripeness of the stain. The following factors also intervene :

(*a*) The fixative employed. Delafield's hæmatoxylin tends to be inhibited by chrome-osmium fixatives and Regaud's fluid, whence the need for longer staining after these—sometimes up to several hours.

(*b*) Tissues which have been preserved, preparatory to embedding, in alcohol for months or years likewise require prolonged staining.

(*c*) Remember that lymphoid tissue, also such organs as contain it in large amounts (*e.g.* spleen and thymus), stains deeply and so rapidly that differentiation is usually essential.

(3) *Time required in Eosin.*—This dye acts more slowly after chrome-osmium, or Regaud fixation (*like* hæmatoxylin), and also after formol fixation (*unlike* hæmatoxylin). This remark does not apply to corrosive-sublimate-formol on account of the mercuric chloride, which greatly facilitates staining with eosin and other acid dyes.

(4) *Use of the Staining Microscope.*—With known material, fixed in a known fixative, the use of the microscope may frequently be dispensed with, provided the period of dehydration be kept approximately constant.

All research material, stained by the above or any other method, should *always* be controlled by the use of the microscope. The total time lost does not amount, for the practised eye, to more than one quarter to one minute, while you are able to bring into the greatest degree of prominence those elements which you wish to observe or study.

2. Staining with Ehrlich's Hæmatoxylin and Eosin

Since Ehrlich's hæmatoxylin is employed in many laboratories its use is mentioned here.

Procedure.—Exactly as for Delafield's stain with the reservation that Ehrlich's stain is less powerful. Consequently a longer treatment —five to ten minutes for most material—is required. Being in acid solution it requires longer to blue it.

Delafield's hæmatoxylin is cheaper to make up, more powerful and precise as a nuclear stain, and more rapid in staining. It is the standard type of hæmatoxylin used in Germany, and it should be used in lieu of Ehrlich's stain for most purposes. The results with Ehrlich's hæmatoxylin are similar to those given by Delafield's hæmatoxylin.

3. Hæmatoxylin (of Delafield or Ehrlich) and van Gieson

The standard method for selectively staining collagen connective-tissue fibres.

PROCEDURE.—Take the sections down to water.

1. Overstain in hæmatoxylin—*i.e.* treat for triple the normal time.
2. Rinse in water.
3. " Blue " the sections. The cell nuclei should be dark blue.
4. Treat three to five minutes with van Gieson's stain (formula, p. 112).
5. Rinse rapidly in water (a few seconds only).
6. Control the degree of staining with the microscope.
7. Dehydrate, clear, and mount in dammar or balsam.

NOTES.—(1) It is essential to overstain your material with hæmatoxylin because it is differentiated by the picric acid component of the van Gieson's stain. Treatment with weak HCl is not required.

(2) Differentiation of the van Gieson is usually not necessary. But should it be needful, proceed thus :

To destain the acid fuchsin (red) component, prolong the washing of the sections in tap water, which, being faintly alkaline, extracts the acid dye.

A rapid rinsing in water, followed by dehydration in the minimum of time, is usually requisite in order not to extract too much of the van Gieson.

To destain the picric acid (yellow) component, a slight increase in the period of dehydration in 70 per cent. and 90 per cent. alcohol will suffice, owing to the solubility of picric acid in alcohol.

(3) *A non-fading substitute for van Gieson.*—Stain for five minutes

in the Ponceau S mixture of Curtis (formula, p. 113), which is used in an exactly similar way to van Gieson. The advantage of this method is that the Ponceau S, in contrast with the acid fuchsin of van Gieson, is perfectly permanent. Its disadvantage is that it is not quite so specific for newly formed connective tissue.

Both the original van Gieson and the substitute stain tissues as follows : Cell nuclei, dark brown or blackish ; collagen fibres, bright red ; red blood corpuscles, muscle, epithelia, and other tissues, a transparent yellow.

4. Safranin and Weigert's Elastin Stain

Weigert's elastin stain (formula, p. 113) is here taken as the specific type stain for elastic connective-tissue fibres. The safranin (formula, p. 112) stains the cell nuclei and brings into evidence the general conformation of the tissues.

PROCEDURE.—Stages 1 to 3 (*a*) of the general scheme. If iodine and " hypo " treatment is necessary, take the section subsequently up through 70 per cent. and 90 per cent. alcohol. It is important to go direct from high grade (90 per cent.) alcohol direct into this stain because it is only soluble—and consequently made up—in alcohol. The frequent introduction of slides taken from water into Weigert weakens the latter by the progressive precipitation of its constituent.

1. Stain twenty minutes (if possible, longer—*i.e.* one hour) in the Weigert *in a stoppered slide jar.*
2. Rinse in 90 per cent. alcohol till no more stain comes out of the sections.
3. Seventy per cent. alcohol.
4. Water.
5. Stain in safranin for five minutes.
6. Rinse in water.
7. (*a*) Dip into 90 per cent. alcohol and (*b*) rapidly examine with the staining microscope. Repeat (*a*) and (*b*) as many times as necessary until the cell nuclei stand out a bright transparent red.
8. Absolute alcohol (rapid).
9. Xylol.
10. Dammar or balsam.

Elastic fibres, dark blue to black ; cell nuclei, bright red ; ground substance of hyaline cartilage, yellow, provided that the safranin be not too much extracted.

Both safranin and Weigert's elastin are perfectly permanent stains.

NOTES.—(1) *Never* pour Weigert's elastin stain on to the sections, as it rapidly evaporates, leaving a precipitate.

(2) You cannot overstain with Weigert.

(3) Remember that while the stained sections are permanent, the Weigert solution is not. Different samples vary enormously in the time they may be kept before losing their staining power. But several months seem to be the average.

(4) You differentiate the safranin during dehydration, which usually needs to be rapid to avoid complete decolorisation of the nuclei. The use of the staining microscope is indispensable during dehydratior

5. Weigert's Elastin and van Gieson

This combination is sometimes useful for demonstrating, in one and the same specimen, both elastic and collagen fibres.

PROCEDURE.—Preliminary stages as for type method No. 4.

1. Stain in Weigert's elastin stain half-hour or longer.
2. Rinse in 90 per cent. alcohol.
3. Pass through 70 per cent. alcohol into
4. Water.
5. Stain in van Gieson three to five minutes.
6. Rinse rapidly in water (a few seconds only).
7. Dehydrate (rapidly).
8. Clear.
9. Mount in dammar or balsam.

Collagen fibres, red ; elastic fibres, blue to black ; red blood corpuscles, epithelia, muscle, etc., yellow.

NOTE.—The Ponceau S substitute for van Gieson (p. 113) may be used instead—usually with advantage.

6. Mallory's Connective-Tissue Stain

A triple stain, useful not only for demonstrating collagen connective tissue (for which purpose it is less specific

than van Gieson), but also for general micro-anatomical purposes.

PROCEDURE.—Fix (preferably) in Zenker's fluid, for use after which Mallory devised this staining method. But excellent results are also obtained after mercuric chloride-formol.

Stages 1 to 4 of the general scheme are applied to sections on the slide.

1. Stain with a 1 per cent. aqueous solution of acid fuchsin one to three minutes.

2. Rinse rapidly in water.

3. Treat with a saturated (*i.e. circa* 1 per cent.) solution of phosphomolybdic acid for half to one minute.

4. Rinse rapidly in water and transfer the section to the following solution in a slide bottle :

Water-soluble aniline blue . .	0·5 grm.
Orange G	2 grms.
Oxalic acid	2 grms.
Aq. dest.	100 c.c.

Stain in the above for two to five minutes.

5. Rinse in water.

6. Dehydrate (rapidly), clear, and mount in dammar or balsam.

Collagen and retiform connective-tissue fibres, blue ; cell nuclei, cytoplasm, elastic fibres (when large), fibrin, neuroglia, smooth muscle, and axis cylinders, red ; red blood corpuscles and myelin sheaths, yellow to orange ; keratin, bright red. The pathological products known as amyloid and hyaline degeneration are stained blue ; so is mucin.

The stain is fairly permanent.

NOTES.—(1) Material fixed in formol or Bouin does not stain as selectively as after fixation in a mercury-containing fixative. But you can improve the staining by placing the sections for a half to one minute in a saturated aqueous solution of mercuric chloride, treating them with iodine and " hypo " and then proceeding with the stain.

(2) It is better to control with the microscope the degree of staining with the acid fuchsin and the aniline blue-orange G solutions. Both these solutions can be differentiated in running tap water.

7. Eosin and Methylene-Blue

A staining method giving results akin to Delafield's (or Ehrlich's) hæmatoxylin and eosin, except that the staining is brighter, sometimes more precise (*e.g.* for Nissl granules), and more transparent.

Micro-anatomically it is useful for thick sections and for the hæmopoietic organs.

Procedure.—Fix in Zenker's fluid, mercuric chloride-formol or some other mercury-containing fixative.

After Bouin or formol it is impossible to get really good results. But treatment of the sections of such material with a saturated aqueous solution of mercuric chloride for one hour or longer, followed by iodine and "hypo," is sometimes helpful.

Stages 1 to 4 of the general scheme.

1. Flood the surface of the slide with a 5 per cent. aqueous solution of eosin. Hold above a Bunsen burner until water vapour just begins to come off. Leave for one to three minutes.
2. Rinse very rapidly in tap water. By now the sections should be very deeply stained.
3. Flood the slide with Borrel's methylene-blue (formula, p. 110) diluted in the proportion of one part of stain to five of aq. dest. Warm above the Bunsen burner and put aside as for the eosin.
4. Rinse in tap water. The sections should be blue-black at this stage.
5. Differentiate in 96 per cent. alcohol to which a little colophonium (0·25 to 0·5 per cent.) has been added.[1] Control the degree of extraction every five to fifteen seconds with the staining microscope.

Both eosin and methylene-blue destain very rapidly, but the former resists the alcohol longer than the basic dye. A point is reached when the cell nuclei are a bright transparent blue, the cell cytoplasm, muscle, etc., a bright pink. Then :

6. Complete dehydration very rapidly in absolute alcohol.
7. Xylol.
8. Dammar or balsam.

[1] As advised by Mallory and Wright.

Cell nuclei, blue; cytoplasm, collagen connective-tissue fibres, and muscle, pink; red blood corpuscles, a bright pink, likewise the oxyphil granules of the eosinophil leucocyte. In course of time the methylene-blue fades somewhat.

NOTES.—(1) The above staining times are mere approximations, since different tissues, fixed in different fluids, each require individual treatment. It is important that the sections should first be grossly overstained in eosin, then in the methylene-blue.

(2) Speed in dehydration and in the control of the degree of differentiation is essential. The colophonium permits of a sharper differentiation.

(3) If the sections are understained or overdifferentiated (which amounts to the same thing), take them down through the alcohols (which will complete extraction of the stain) to water. Then start again.

(4) A good sample of water-soluble eosin is essential.

8. Mann's Methyl-Blue-Eosin

An extremely easy stain to use; often useful for low-power observations. For critical, high-power work, generally too diffuse. Of use in the study of the relations and development of blood vessels owing to the brilliant red stain acquired by the red blood corpuscles.

PROCEDURE.—The method works after any fixative, but the best results are obtained after those containing mercuric chloride.

Stages 1 to 4 of the general scheme.

1. Stain fifteen to thirty minutes to overnight.
2. Rinse well in water.
3. Differentiate in 70 per cent. alcohol to which 5 drops of a concentrated aqueous solution of orange G have been added to every 5 c.c. of the alcohol. Control the extraction of the stain with the staining microscope.[1]
4. Clear in xylol.
5. Mount in balsam.

Nuclei, blue; karyosomes, dark blue; plasmosomes, red; basophil cytoplasm, blue; oxyphil cytoplasm and oxyphil granules, red.

[1] This method of differentiation is due to Dobell.

NOTES.—(1) Fifteen minutes generally suffices for staining after all fixatives except chrome-osmium. For the latter, stain for several hours or overnight.

(2) Differentiation—other than a slight decolorisation in tap water—is often unnecessary.

9. Masson's Hæmatoxylin-Erythrosin-Saffron Method

This triple stain gives excellent results after most fixatives. It has the advantage over hæmatoxylin and eosin that it gives a much better differentiation of most cells and tissues. This is particularly the case with high-power observations. Collagen fibres are stained a different colour from muscle (yellow and pink respectively). The method is particularly good after fixation in Bouin's fluid, Zenker-formol and mercuric chloride-formol. It may be used as a routine stain for much material in preference to hæmatoxylin and eosin.

Disadvantages: (1) Masson's stain requires about five to ten minutes longer than hæmatoxylin and eosin. (2) One of the solutions—the saffron—does not keep indefinitely.

PROCEDURE.—Stages 1 to 4 of the general scheme are applied.

1. Stain in hæmatoxylin of Delafield, Ehrlich, or Heidenhain.
2. Blue the sections. Collagen fibres should not be stained; if this has occurred, differentiate in weak hydrochloric acid.
3. Treat with 1 per cent. erythrosin (formula, p. 111) for five minutes (often two to three minutes suffices).
4. Rinse rapidly in water.
5. Differentiate in 70 per cent. alcohol for a few seconds or minutes till collagen fibres are colourless or nearly so.
6. Rinse in water.
7. Treat with Masson's saffron (formula, p. 111) for five minutes.
8. Rinse rapidly in distilled water.
9. Dehydrate rapidly in absolute alcohol.
10. Xylol.
11. Dammar or balsam.

Nuclei, blue (grey to black after Heidenhain); cytoplasm, very selectively stained in various tones of red; muscle, pink; elastic fibres (if not too fine), also pink; collagen fibres, yellow. The stain is as permanent as hæmatoxylin and eosin. It must not be used as a substitute for van Gieson, as fine collagen fibres are not as selectively stained as by a specific method.

NOTE.—Dehydration must be rapid and absolute alcohol should be used. If you begin dehydration in low-grade alcohol the saffron will be washed out.

Variant.—You can stain elastic fibres by Weigert's stain before proceeding with the hæmatoxylin. Then as above.

"TYPE" CYTOLOGICAL STAINS

Under this heading are grouped those staining methods which are especially useful for bringing into evidence the components of the nucleus and cytoplasm rather than the different tissue layers.

As with fixation, so with staining: certain cytological staining methods can sometimes be used micro-anatomically and *vice versa*.

1. Heidenhain's Iron-Hæmatoxylin

This method, invented by M. Heidenhain in 1892, remains the standard cytological stain. It is applicable to both nuclear and cytoplasm components. After suitable fixation, it may be used to demonstrate the following elements: chromatin; nucleoli (karyosomes and plasmosomes); nucleolini; the nuclear membrane; centrosomes; mitochondria; yolk; various secretory products; the ground cytoplasm.

PROCEDURE.—Stages 1 to 4 of the general scheme, using iodine and "hypo" if necessary.

1. Place the sections in a slide dish of a 5 per cent. solution of iron alum (formula, p. 107). Leave therein for one-half to twenty-four hours. (The suitable duration of this bath is discussed in the "NOTES.")

2. Rinse in water.

3. Place the sections in a slide dish of 0·5 per cent. solution of hæmatoxylin (formula, p. 108). Leave therein for a time at least equal to the duration of the iron-alum bath.

4. Rinse in water.

5. Differentiate the sections in the iron-alum solution. Control this process with the staining microscope by taking the slide out of the iron alum, dipping it in water, and examining with the 8 mm. ($\frac{1}{3}$ inch) or 4 mm. ($\frac{1}{6}$ inch) objective. Repeat the process until the sections are suitably destained. Then :

6. Transfer them to a slide dish and wash in a gentle stream of running water for two to five minutes. The thicker the sections the longer the washing, the aim of which is to remove all traces of iron alum.

7. Dehydrate at your leisure. Iron-hæmatoxylin is insoluble in alcohol.

8. Clear in xylol.

9. Mount in dammar or balsam.

The tissues are in tones of black and grey according to the degree of extraction of the stain. Red blood cells, deep black.

NOTES.—(1) *The duration of the iron alum and the hæmatoxylin baths.* Almost entirely dependent on the fixative employed.

After fixation in *formol, mercuric chloride-formol, Bouin,* and *Carnoy,* the maximum intensity of staining is attained after one-half to one hour in the mordant, and an equal (or slightly longer) time in the hæmatoxylin.

Zenker-fixed material may require a slightly longer treatment because chrome salts tend to inhibit hæmatoxylin staining.

Regaud's formol-bichromate requires from three to twelve hours in the mordant and stain. For staining mitochondria by this method the best results are obtained by twelve hours' treatment in iron alum and twelve hours in the hæmatoxylin.

Flemming's fluid (with or without acetic acid), *Champy's fluid, and all other chrome-osmium fixatives* : twelve hours in the iron alum and twelve hours (or longer) in the hæmatoxylin.

Sections are adequately stained when, on being taken out of the hæmatoxylin bath, they are a homogeneous jet-black, and show microscopically no cellular detail at all. This rule applies to all fixatives.

(2) *Differentiation.*—Since Heidenhain's iron-hæmatoxylin can only be used regressively, the success of the stain depends almost entirely

on its accurate differentiation. Beginners will find it advantageous to differentiate in a 2½ per cent. solution of iron alum.

The explanation of the use of the mordant (iron and ammonium sulphate) as a decolorising agent in Heidenhain's iron-hæmatoxylin method is that, in addition to being a salt of a metal, and thus forming a lake with hæmatoxylin, it is an oxidising agent. When the over-stained tissues are taken from the hæmatoxylin bath and put into the mordant, the latter, by virtue of its oxidising power, converts the hæmatoxylin into a soluble, uncoloured compound, which diffuses into the solvent.

By varying the time of differentiation it is possible, after suitable fixation, to stain both nuclei and cytoplasm, or nuclei alone. The stain first comes out of the ground cytoplasm, then out of the cytoplasmic constituents (*e.g.* mitochondria, provided, of course, that these have not been destroyed by the fixative), and, lastly, out of the nuclear (chromatinic) constituents.

No time can be given for the duration of differentiation. The only guide is the constant use of the staining microscope. All that can be said is that a tissue of average density, when differentiated to demonstrate only the nuclei, is usually a pale grey, when destained to show mitochondria and other cytoplasmic elements, a dark grey.

Be careful not to mistake red blood corpuscles for cell nuclei, as the hæmoglobin of the former combines very energetically with the iron-hæmatoxylin. Decalcified bone and keratin also retain this stain very energetically.

(3) *The thinner the sections the better the results*, though, contrary to the current statement, quite useful results may be obtained with sections of 10 μ, or even more in the case of many tissues.

(4) If you overdifferentiate the sections, place them again in the hæmatoxylin until they are jet-black. Then redifferentiate.

(5) *The advantages of iron-hæmatoxylin are :*

(*a*) It works after any fixative. Material which has lain for years in alcohol and which is refractory to other stains may often be stained by this method.

(*b*) It is one of the few really permanent stains known. It never fades—provided the iron alum is properly washed out of the sections after differentiation.

(*c*) It stains in tones of black and grey. This means a reduction of eye-strain during high-power observations, good results with micro-photography, and an accurate reproduction of the images seen with the microscope in Indian ink or wash sketches.

(*d*) By altering the duration of differentiation it is possible to stain either nuclear or cytoplasmic constituents.

(*e*) Iron-hæmatoxylin can be combined with a large number of counter-stains.

2. Combined Staining with Heidenhain's Iron-Hæmatoxylin and other Stains

Practically any counter-stain may be used before or after iron-hæmatoxylin. For many cytological purposes no counter-stain is necessary. For micro-anatomical work, however, a fully differentiated (*i.e.* largely nuclear) stain is indicated, so that a counter-stain is here an advantage.

The following combinations will be found useful :

Iron-Hæmatoxylin and Eosin.—Use exactly as with Delafield's hæmatoxylin.

Iron-Hæmatoxylin and Orange G. — Keep a saturated solution of this in absolute alcohol in a staining jar. Pass through 70 per cent. and 90 per cent. alcohol into this solution, which will stain and dehydrate simultaneously. Usually two to three minutes are sufficient. If overstained, either differentiate in alcohol or in running tap water. The latter takes the stain out more rapidly, since orange G is more soluble in it than in alcohol. A transparent, yellow, or pale orange stain. Permanent.

Iron-Hæmatoxylin and van Gieson.—Use as with Delafield's hæmatoxylin (see p. 129), remembering, however, that you need only very slightly overstain with the hæmatoxylin, since it is only slowly discolorised by the picric acid component of the van Gieson.

The van Gieson substitute (see p. 129) may often be used with advantage in place of the original van Gieson. Excellent micro-anatomical slides for demonstration or projection purposes may be prepared in this manner, with the great advantage that they do not fade.

Iron-Hæmatoxylin and Lichtgrün.—Pass the sections from 70 per cent. alcohol into a saturated solution of lichtgrün in 90 per cent. alcohol in a slide bottle. Stain therein from a few seconds to two minutes. Rinse in, and, if necessary, differentiate in 90 per cent. alcohol from a drop-bottle (to

avoid contamination of the slide bottle of 90 per cent. alcohol). Complete dehydration in absolute alcohol. An extremely transparent cytoplasmic stain, but unfortunately one which fades after several months.

NOTE.—All these counter-stains tend to be inhibited by chrome-osmium fixatives. Whence the need, after these, for staining for the maximum times prescribed, and sometimes even longer.

3. Regaud-Kull and Formol-Kull Methods

The above are both variants of the well-known Champy-Kull technique. The only difference is the difference of the fixative : better fixation is obtained with Regaud's fluid or formol than with the Champy fixative in the case of mammalian tissues.

The last of these variants is due to Mr. J. R. Baker, whom I thank for allowing me to mention as yet unpublished investigations. The value of Baker's technique lies in the fact that it enables even formol-preserved material of several months' standing to be selectively stained. The Champy-Regaud and Formol-Kull methods give excellent triple-stained sections for demonstrating certain cytoplasmic inclusions, and notably mitochondria. If the material is osmicated—either during or after fixation—fat globules are also demonstrated.

PROCEDURE

1. *Fix* in *one* of the following fluids :

(*a*) *Champy's fluid* or *Flemming without acetic* (see p. 40). Not recommended for mammalian tissues. Twelve to twenty-four hours' fixation.

(*b*) Regaud's fluid (formula, p. 40). Two to four days' fixation. Recommended.

(*c*) Four or 5 per cent. formalin in 0·9 per cent. NaCl. Two to four days' fixation or longer. Also good.

Then proceed as follows for all three fixatives :

2. Wash in distilled water for half an hour.

3. Transfer to the *Pyroligneous-chromic acid mordant* for approximately twenty hours.

Pyroligneous acid . . .	33 c.c.
1 per cent. chromic acid . .	66 c.c.

4. Rinse half an hour in distilled water.

5. Transfer to a 3 per cent. solution of potassium bichromate. Leave therein for three days.

6. Wash in running tap water for twenty-four hours.

7. Dehydrate.

8. Clear in xylol or benzol (rapidly).
9. Embed in paraffin wax.
10. Make thin sections (5 μ or less).

} According to the standard technique.

11. Flood the sections with Altmann's acid fuchsin (modified as in formula, p. 110). Warm above a Bunsen burner until steam comes off. Put aside for six minutes.

12. Rinse rapidly in aq. dest.

13. Counter-stain in a 0·5 per cent. aqueous solution of toluidin blue for one-half to two minutes. The aim of this is to stain the nuclei. (1 per cent. aqueous thionin does as well.)

14. Rinse in aq. dest.

15. Transfer to a slide bottle of 0·5 per cent. of aurantia in 70 per cent. alcohol. Control every few seconds with the microscope. The time in the aurantia varies (usually from five to thirty seconds). This dye in alcohol extracts the acid fuchsin from the ground cytoplasm partly by solution, partly by substitution.

16. Dehydrate *rapidly* in absolute alcohol.

17. Clear in xylol.

18. Mount in dammar or balsam.

The stain is not permanent owing to the fading of the acid fuchsin component.

Chromatin, blue ; plasmosomes, red ; mitochondria, red ; fat (if osmicated), black ; yolk (if osmicated), yellow to black according to its fat content ; red blood corpuscles, red ; ground cytoplasm, pale yellow.

Notes.—(1) A good accessory method to iron-hæmatoxylin, but one which needs some practice to manipulate successfully. The Regaud fixation, when followed by the Kull stain, has given me excellent mitochondrial preparations.

(2) It is important to stain very deeply with the acid fuchsin owing to its destaining (partly) in the toluidin blue or thionin, and in the aurantia.

(3) I get the best results by heating the acid fuchsin to 60° to 70° C. in a small test-tube before pouring it over the sections.

(4) If you wish to fix in Regaud's fluid but also to preserve fat, you can place the tissues in 2 per cent. osmic acid for twelve to twenty-four hours after fixation. Then proceed with stage 3 (pyroligneous-chromic acid).

For other mitochondrial methods, see p. 231.

4. Cajal's Method for the Golgi Elements

Of the various methods for demonstrating the Golgi elements, the above, and particularly Da Fano's modification of it, is the surest and most selective in action. Special fixation is essential. *You cannot apply this technique either to material fixed by the ordinary methods or to sections on the slide.*

The principle of this technique is the same as for certain other impregnation methods, used exclusively for the central nervous system. Although Cajal first applied his technique to the Golgi elements of the nerve cell, he found that it could be extended to practically all the other tissues.

This method, also the Cajal technique for the demonstration of neurofibrils, may be termed "photographic."

The elements to be demonstrated are submitted to the following processes: (*a*) *fixation*; (*b*) *permeation* with a solution of silver nitrate; (*c*) *reduction* of the silver over the elements to be demonstrated by means of a modified photographic developer; (*d*) *toning*—applied to sections on the slide. As with photography so here: a partial substitution of gold in the place of the silver salt is induced by placing the sections in a solution of gold chloride. To the latter sodium hydrogen sulphite ($NaHSO_3$) is frequently added, so as to dissolve any unreduced silver and thus obviate its eventual precipitation.

Procedure.—The tissues must be fixed immediately after death, or, at the latest, not more than three hours afterwards (at room temperature). Autolysis of the Golgi elements occurs fairly rapidly after death.

1. Fix medium-sized (*i.e.* not thicker than 5 mm.) pieces of tissue ten to fourteen hours in Cajal's uranium nitrate-formol (formula, p. 114).

For the original Cajal fixative, Da Fano's modification may be advantageously substituted. In this case fix three to eight hours in cobalt nitrate-formol (formula, p. 114).

2. Rinse ten to thirty seconds in distilled water.

3. Impregnate with a 1·5 per cent. solution of silver nitrate at room temperature for thirty-six to forty-eight hours.

4. Rinse in distilled water as above.

5. Reduce for one to two hours in the reducing fluid (formula, p. 114).

6. Wash for five minutes in running tap water.

7. Dehydrate in ascending grades of alcohol, bearing in mind that Penfield has shown that the duration of the baths of 90 per cent. and especially absolute alcohol should be as short as possible—*i.e.* one to two hours in each. Clear and embed in paraffin in the usual manner.

8. Make sections on the slide.

9. You may : (*a*) mount direct in dammar or balsam without toning after dissolving the paraffin in xylol. It is better to rinse the sections rapidly in absolute alcohol in order to remove all traces of the glycerine of the adhesive mixture used for sticking down the sections.

(*b*) Tone the sections by pouring over the slide equal parts of the two toning solutions (formulæ, p. 115). Mix them in a small tube just before use. Control with the microscope. The yellow coloration of the cytoplasm will turn greyish in (usually) five to ten minutes. Toning is then complete.

Arrest toning by washing in running water. Dehydrate, clear, and mount in balsam.

(*c*) Counter-stain the sections after toning them. Safranin gives good results. Use as described on p. 130. Delafield's (or Ehrlich's) hæmatoxylin are also applicable, but *not* the iron-hæmatoxylin of Heidenhain, since the alum solution completely destains the Golgi elements.

The Golgi apparatus appears under the 4 mm. ($\frac{1}{6}$ inch) objective as a black network or system of rodlets. Ground cytoplasm, grey (after toning), yellow or light brown if the sections are not toned. Mitochondria are frequently impregnated golden to dark brown. Impregnation of the myofibrillæ of unstriated muscle sometimes occurs.

In addition to the above, certain elements—mostly of an enigmatical nature—have been noted to undergo an intense impregnation. They comprise :

(i) A spiral filament around the nuclei of unstriated muscle fibres (Rio Hortega).

(ii) A spiral intranuclear rod in the ovarian egg-cells of mammals (Rio Hortega).

(iii) One or more granules or rodlets (="Nucleolini") inside the nucleoli (noted in several species by Rio Hortega and Carleton). A special modification of the Cajal method is often necessary to demonstrate these bodies, which are also stainable in iron-hæmatoxylin after very careful differentiation with a water-immersion objective.

It follows from the above that an absolutely specific impregnation for the Golgi elements does not, at present, exist, and that care must be taken in their identification.

NOTES.—(1) A successful impregnation of the Golgi elements is primarily dependent on the correct degree of (*a*) fixation and (*b*) silvering. This can only be ascertained by the trial-and-error method. The best plan is to fix a number of pieces of tissue and to take one (or more) out of the fixative (and silver bath) at intervals. *E.g.* fixation (Da Fano's formula) for three, six, and eight hours ; impregnation in the silver bath for thirty-six and forty-eight hours. Over-fixed or over-impregnated tissues are (in section) very dark and show a black precipitate ; under-fixed or under-impregnated ones show no Golgi elements. Embryonic material requires a shorter treatment than adult.

(2) Use a large volume of fixative and especially of silver nitrate ; *e.g.* 50 c.c. of the former and 100 c.c. of the latter for pieces of tissue of average size (*i.e.* not more than 5 mm. in thickness). Change the silver nitrate solution twice during impregnation, and use a glass-stoppered or glass-covered vessel to avoid precipitation of the silver by contact with a cork.

(3) The silver nitrate solution must be fairly fresh. If the stock solution shows a black precipitate of silver oxide at the bottom of the bottle, throw it away and make it up afresh.

(4) Contrary to the usual statement, there is no need to reduce for longer than two hours, as the maximum of reduction occurs very rapidly. The reducing fluid goes brown after a few weeks, but is still efficacious.

See also the original method of Golgi (p. 243).

5. Pyronin-Methyl-Green

Of use for determining the oxyphility or basophility of the cell components. Although a combination of two *basic* dyes, the affinity of methyl-green for chromatin and other basophil bodies is so marked, and the proportions of the two dyes are such, that this seemingly irrational stain is one of the best for determining whether cell or tissue components are basophil or oxyphil.

PROCEDURE.—Fix in absolute alcohol, mercuric chloride, or mercuric chloride-acetic acid. After Flemming, staining must be very prolonged (several hours or overnight). Avoid formol and fixatives containing it. For work on the oxyphility and basophility of the cell constituents, fixation in absolute alcohol gives the most trustworthy staining reactions. But many tissues are grossly shrunken by it.

1. Take the sections down to distilled water.
2. Stain with Unna's pyronin-methyl-green (formula, p. 112) for five to thirty minutes.
3. Rinse in distilled water.
4. Blot the sections.
5. Dehydrate rapidly in absolute alcohol.
6. Xylol.
7. Dammar or balsam.

Chromatin reticulum and karyosomes, bluish green; plasmosomes, red; oxyphil constituents of cytoplasm, red; basophil ones, green. Cytoplasm of plasma cells, bright red. Bacteria are also vividly stained red. Ground substance of hyaline cartilage, yellowish red.

The methyl-green fades in course of time.

NOTES.—(1) The requisite amount of differentiation is effected by the absolute alcohol. It is safer only to dehydrate for a few (five to ten) seconds, and examine the slide after clearing in xylol under the staining microscope. If necessary, you can differentiate again for a short time in alcohol.

(2) All chrome-osmium material requires prolonged staining (preferably several hours).

B. STAINING OF CELLOIDIN SECTIONS

Celloidin sections, as already explained, are as a rule cut individually, and stored in 70 per cent. alcohol until required for staining.

1. General Procedure

The same general principles apply equally to the staining of both paraffin and celloidin sections, with but one exception : *celloidin is soluble in absolute alcohol. Consequently this substance must not be used for dehydration.*

The following methods of dehydration and clearing are recommended :

I. *The Creosote Technique*

The details of this method were given me by Mr. W. Chesterman ; it has given me flatter sections than any other method I have tried.

1. Dehydrate in ascending grades of alcohol up to 90 per cent.

2. Treat with 95 per cent. alcohol for a few minutes (two to three).

3. Place the sections in the following fluid for not longer than five minutes :

Xylol . . .	Equal parts.
Chloroform . .	
Absolute alcohol .	

4. Transfer the sections to a dish of pure beechwood creosote. If wrinkled, they will rapidly flatten out in this. Do not prolong treatment in this for more than seven or eight minutes, as celloidin is slightly soluble in creosote.

5. Place a slide beneath the section or sections and float them on to it. When in position, slowly take the slide out, holding the section in place with a needle held in the other hand.

6. Blot with cigarette or toilet paper.

7. Pour xylol on to the slide ; drain it off and repeat the process once or twice. Do this quickly, as prolonged action of xylol will cause the sections to crease.

8. Blot rapidly.
9. Mount in dammar or balsam.

II. *Xylol-Acetone Method*

1. Dehydrate in ascending grades of alcohol to 90 per cent.
2. Place in 95 per cent. alcohol for two to three minutes.
3. Complete dehydration in the following mixture :

Acetone (water-free) . .	1 part.
Xylol	4 parts.

4. Pure xylol, etc., as above.

A rapid method, but inferior to I.

III. *Other clearing media*, which will remove water from sections after treatment with 95 per cent. alcohol, are :

Origanum oil.
Bergamot oil.
Carbol-xylol (phenol cryst., 25 grms. ; xylol, 75 c.c.).

The latter clears from even 90 per cent. alcohol. But it has the disadvantage of attacking most of the basic aniline dyes on account of its acidity. Sections should always be rinsed in xylol after carbol-xylol.

The celloidin mass, being perfectly transparent, should *not* be removed, a procedure which has the additional advantage of keeping together the tissues in the sections.

2. Methods of Manipulation

(i) *The Watch-Glass Method*

The most frequently used. Not suitable for serial sections.

You arrange a series of watch-glasses, shallow capsules, or Petri dishes on the staining bench. These you partially fill in the correct sequence with the solutions and stains you intend to employ.

Thus, to stain a formol-fixed celloidin section in Delafield's

hæmatoxylin and eosin, a series of vessels containing the following substances are arranged in the order of their use :

(Sections preserved in 70 or 80 per cent. alcohol.)
50 per cent. alcohol.
*Water.
Delafield's hæmatoxylin.
*Water.
(Dilute HCl to differentiate the hæmatoxylin, if necessary.)
Tap-water substitute (to blue the nuclei).
Eosin.
*Water.
70 per cent. alcohol.
96 per cent. alcohol (rapid) . } Note difference in technique
Acetone-xylol or other mixtures (*vide supra*) . . } as compared with dehydration of paraffin sections.
Xylol.

* = *The same dish is used throughout.*

To manipulate the section, lift it by the edge of the celloidin mass on to a large spatula.

(See also " NOTES " at end of this section.)

(ii) *Lee's Albumen Method*

This, and the following technique, are methods whereby the sections are stained on the slide. They are therefore especially applicable to serial work.

1. Take the sections, one by one, down to distilled water.
2. Then press each one down on to a slide, the surface of which has been smeared with the finger with a tiny drop of the concentrated glycerine and albumen solution.
3. Allow to dry at room temperature.

Then treat according to the standard technique for celloidin sections. It is safer *not* to dissolve the celloidin mass from such sections.

(iii) *The Collodion Film Method*

Suitable for class or serial purposes, since a number of sections may be treated simultaneously.

Remove the emulsion from a photographic film by a thorough cleansing in hot water. Allow to dry. Cut into strips of suitable dimensions. Then proceed as follows :

1. Pass the sections through 96 per cent. alcohol.
2. Arrange them in order on the strip of film.
3. Add a few drops of absolute alcohol to the surface of the film. (This partially dissolves the collodion so as to weld sections and film into one transparent mass.)
4. Proceed with staining or store the sections either in 70 to 80 per cent. alcohol or in cedar oil (after dehydration in the xylol-acetone mixture).

NOTES.—(1) Nearly all staining methods are applicable to celloidin material, provided the sections are not too thick. Remember that Heidenhain's iron-hæmatoxylin, or the Champy-Kull mitochondrial method, for instance, are intended for thin (*i.e.* 6 μ) sections ; whereas the standard thickness for celloidin sections is about 12 μ. Hence their use is not indicated with celloidin material.

(2) Never use the celloidin method unless essential ; not only are the processes of inclusion and section cutting far longer than with the paraffin technique, but staining the sections is also more laborious.

(3) Since the inclusion mass is retained during staining of celloidin sections—in contrast with paraffin ones—the permeability of the tissues to reagents is somewhat diminished. It is therefore necessary to allow dehydrating and staining solutions to act rather longer than with paraffin sections.

C. STAINING FROZEN SECTIONS

Frozen sections, being free, are manipulated like celloidin ones, but with even greater care, owing to the absence of an embedding mass (unless gelatine-embedded).

Either transfer the sections very carefully with a spatula from one solution to another, or pipette off the solutions from the sections as they lie in a shallow watch-glass.

They are transferred from the microtome knife to a shallow dish of distilled water or 50 per cent. alcohol. From either of these media they may be stained by most standard staining methods—*e.g.* Delafield's hæmatoxylin and eosin. If it be particularly desired to avoid shrinkage, mount the sections in glycerine jelly. Transference through the alcohols and

xylol causes some shrinkage, which may be greatly reduced by the following modification of Schmorl :

1. Partially dehydrate in 96 per cent. alcohol.
2. Complete dehydration, and clear, in carbol-xylol (formula, p. 147).
3. Mount in dammar or balsam.

NOTE.—Unfortunately many basic dyes are decolorised by the acidity of this clearing mixture.

For the special staining methods for fat, see p. 169.

For rapid diagnosis of sections, stain in Delafield's hæmatoxylin and eosin, and mount direct in glycerine jelly. But this stain fades in course of time in glycerine jelly mounts.

PART III

ACCESSORY METHODS OF EXAMINATION

CHAPTER X

DARK-GROUND (OBLIQUE) ILLUMINATION

1. Principle—2. Uses—3. Adjustment of the Dark-Ground Condenser —4. Notes.

1. PRINCIPLE

The customary method of illumination of microscopic object is by transmitted—*i.e.* direct—light. This means that the light rays are reflected from the mirror, concentrated by the

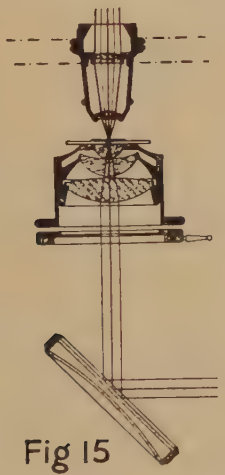


Fig. 15.—Path of Light Rays through a Three-Lens Abbe Condenser. (After Zeiss.)

An illustration of direct illumination of the object.

condenser, and thence passed directly through the object (see Fig. 15).

In the dark-ground method of illumination the path of the light rays are quite different : no direct rays of light enter

the objective. All such objects or particles as can be seen are visible *not* because they are traversed by light rays, but because of the diffraction of the light rays around them.

Diffraction is a peculiar phenomenon which may be expressed, in Bayliss's words, as "the property of waves (of light) to bend round corners." The commonplace observation of the dancing dust particles in a beam of sunlight in a darkened room is a diffraction phenomenon. It is due to the fact that the light waves bend around each particle, and, in so doing, they form a little diffraction halo around it. Note, moreover, that in order to see the particles in such a beam it is necessary that the line of vision be at right angles—or at

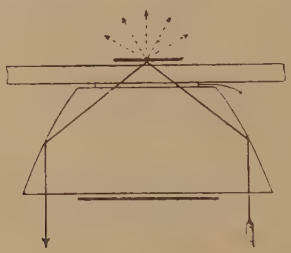


FIG. 16.—PATH OF RAYS OF LIGHT THROUGH THE DARK-GROUND (PARABOLOID) CONDENSER OF ZEISS. (After Zeiss.)

No direct rays enter the objective but only those which have been diffracted, refracted, or reflected.

any rate obliquely directed—with regard to it. If you face the beam you see nothing except the dazzle. In other words, it is important to avoid, so far as is possible, getting any direct light rays into your eyes, since these will mask the diffracted rays formed around the particles.

There are too many types of dark-ground condenser to undertake their individual description, but the principle may be illustrated by taking the paraboloid condenser of Siedentopf—who, with Szigmondy, has done so much for the development of dark-ground illumination.

As shown in Fig. 16, this condenser consists of a single lens, its upper and lower surfaces plane, the sides slightly convex. Applied against the lower surface is a circular black disc. The whole is substituted for the ordinary

condenser in the substage. Between condenser and slide is a layer of cedarwood oil. Now, suppose that the fluid beneath the cover-slip is optically homogeneous—*i.e.* devoid of particles down to, and including, colloidal ones.

The path of a pencil of light will be as in the continuous arrow line in Fig. 16; it will undergo total reflection, and the field will consequently appear black and empty. But if particles are present between cover-slip and slide, they will be brilliantly lit up by the indirect rays of light which will produce bright diffraction haloes around them. (See dotted arrow lines in Fig. 16.) The apparent paradox of making ultra-microscopic particles visible is due to the fact that it is not the particles themselves but the diffraction discs around them which are seen. Even "large" objects—for instance, red blood corpuscles—often show this bright diffraction line at their periphery.

Upon this principle of a brilliant oblique illumination of the object, so that no direct, but only diffracted and refracted rays, enter the objective, are based all forms of dark-ground condenser and of ultra microscope.

A dark-ground condenser is constructed so that reflection of the light rays takes place a definite distance above it—*i.e.* at the interface between the top of the cover-slip and the air (or oil, in the case of an oil immersion). This means, in practice, that the thickness of the slide and cover-slip—and especially of the former—are of importance if clear images are to be obtained.

All dark-ground condensers are calculated to give the optimum results with a slide of a definite thickness. When using these instruments, you must bear this in mind. A slide of 1 *mm. thickness gives good results with nearly all makes of dark-ground condenser.* The limits of thickness of the slide are usually indicated in the box supplied with the dark-ground condenser. Or they may be found in the maker's catalogue.

2. USES

The dark-ground condenser is especially used for the detection of spirochætes, trypanosomes, and other micro-organisms in body fluids.

Recently it has been extended to the study of the colloidal particles in protoplasm.

It is essentially a method of examination of fresh preparations rather than of sections or fixed material. Owing to the diffraction haloes formed around objects by this method it is essential, especially when making original observations, carefully to control all appearances seen with the dark ground by examination of fresh (and stained) preparations of such material with ordinary (direct) illumination.

3. ADJUSTMENT OF THE DARK-GROUND CONDENSER FOR USE

Good results depend on the correct focusing and centring of the illuminant, the bull's-eye condenser, and also of the dark ground itself.

1. **The Illuminant.**—A fairly powerful lamp is a necessity with most forms of dark ground. Failing any other type of lamp, a 50 c.p. "Mazda" bulb or an incandescent gas burner give quite good images. When much dark-ground work is done, a Nernst lamp, a "Pointolite," or a small arc lamp should be employed. In wild places, where gas and electricity are not available, an acetylene lamp is a satisfactory substitute.

In the forms of Pointolite, Nernst, and arc lamps sold for microscopic work, a bull's-eye condenser forms part of the lamp. When working with an ordinary electric bulb or incandescent burner, some form of bull's-eye condenser should also be interposed between the source of light and the mirror of the microscope. The aim of this is uniformly to cover a given area of the mirror with a disc of light. The distance between illuminant and mirror should be 20 to 40 cm. (8 to 16 inches), and the condensing lens is placed midway between the two. If this lens forms part of the mounting of the illuminant, the latter should be placed close to the microscope (*e.g.* 20 cm. from it).

2. **Centring and Focusing the Beam of Light on the Mirror.**—Adjust the condensing lens so that it throws a beam of light on to the mirror. *Always use the plane surface of*

this for all dark-ground work. Then adjust the distance between the lamp and the condensing lens until a bright circle of light falls exactly in the centre of the mirror. The size of the circle required to give the best results varies with different makes of dark ground ; generally it should be about the size of a sixpenny piece to that of a shilling (*i.e.* about 2 cm. in diameter). If you have trouble in seeing the disc of light, place a piece of white paper on the mirror.

3. **Centring the Dark-Ground Condenser.**—This is very important, as, unless the centring is correct, aberrant rays of light will enter the microscope and destroy the dark-ground effect. It is assumed that you possess a centring substage dark-ground condenser.[1] On the upper surface of nearly all dark-ground condensers one or two circles are engraved. Focus one of these with the 16 mm. ($\frac{2}{3}$ inch) objective ; by means of the centring screws of the substage, adjust the condenser so that the engraved ring is central with regard to the field in the ocular. Now look at the top of the dark-ground condenser ; you will see a dark disc partially covered by a ring of light. Adjust the mirror forwards and sideways until this ring of light is central and equal in thickness all round. Then lower the condenser by a few millimetres.

4. Add several drops of *thin* cedarwood oil (or distilled water) to the top of the dark-ground condenser. The former is preferable. Place the slide so that one edge lies roughly above the centre of the dark-ground condenser. Then gently rack the latter up until the oil has met the under surface of the slide, which is then moved so as to cover the condenser. The idea of all this is to avoid air-bubbles in the oil, which, if large or numerous, greatly impede observation.

5. First focus the object with the 16 mm. ($\frac{2}{3}$ inch) objective.

[1] Some makes of dark-ground condenser form part of a flattened mount which is clipped on to the stage of the microscope. With these, centring is effected (more or less) by pushing the condenser about the stage and finally clamping it.

Others fix in the substage in place of the ordinary condenser, but have no centring device.

Both these types, and particularly the latter, are bad.

The cells, etc., should appear a brilliant white on a black background. Generally, the condenser needs to be slightly raised or lowered in order to get a uniform illumination of the field. The layer of oil between slide and condenser must always be maintained. If not, total reflection of the rays will ensue, and no light will enter the objective. A slight readjustment of the height (*i.e.* focus) of the dark-ground condenser is usually necessary each time the objective is changed.

NOTES

1. **Use of the Oil Immersion.**—Before using a 2 mm. ($\frac{1}{12}$ inch) or higher-powered lens, a specially constructed stop, supplied by the maker, must be inserted in the mount of the objective. This is because the wide numerical aperture of a 2 mm. oil immersion admits light rays, in addition to those reflected or diffracted from the object under study, to enter the microscope. Unless these peripheral rays are eliminated by the stop, the dark-ground effect cannot be realised.

The 3·45 mm. ($\frac{1}{7}$ inch) oil immersion, now made by several firms specially for blood and dark-ground work, does *not* need a stop because of its low numerical aperture.

2. **The High Dry Objectives** are also improved in performance by the use of the stop. Generally speaking, the higher the objective the less black the background (unless a stop is inserted) owing to the increase in numerical aperture in the higher series. This remark applies particularly to apochromats. Compensating oculars should always be used with these lenses. A certain greyness of the background appears to be inherent to the use of many apochromats for dark-ground work. But it does not hinder observation.

3. **Thickness of the Preparation.**—The slide, as mentioned, must be within certain limits of thickness. The layer of fluid in which the cells or micro-organisms are being examined should be as thin as possible and free from air-bubbles, which, if numerous, give rise to troublesome reflections. The cover-slip must also be thin.

4. **If the Slide is Too Thin.**—In such a preparation the background is black, but the cells, etc., are not illuminated.

This can usually be corrected by lowering the dark-ground condenser.

5. **If the Slide is Too Thick.**—The field is greyish ; illumination and visibility of cells is poor. Try raising the condenser to its maximum. If this does not improve matters, make a fresh preparation on a slide of the proper thickness. When dark-ground observations are often undertaken, a supply of measured slides should be kept.

6. **Scratches or Grease Marks** on slide or cover-slip show up all too clearly with dark-ground illumination. Absolutely clean slides and cover-slips must be used.

7. **For High-Power Observations** — *e.g.* above 500 diameters—either use a 2 mm. ($\frac{1}{11\frac{1}{2}}$ inch) oil immersion provided with a stop, or a 4 mm. ($\frac{1}{6}$ inch) apochromat plus stop with a high-powered compensating ocular.

CHAPTER XI

HISTO-CHEMICAL TESTS

Introduction.
Making a Fresh Preparation.
1. Identification of Adrenalin: Cramer's Osmic Acid Method (sections only).
2. Identification of Calcium Salts: (1) Fresh Preparations—(2) Sections: Von Kossa's Silver Nitrate Method; Purpurin Method.
3. Identification of Chromatin: (1) Fresh Preparations: Methyl-Green—(2) Enzyme Tests for Chromatin—(3) Sections: Pyronin-Methyl-Green.
4. Identification of Fat and Lipoids: (1) Value of Histo-Chemical Tests for Fat—(2) The Scharlach R Technique (Fresh Preparations and Sections)—(3) Methods of Osmication (Fresh Preparations and Sections)—(4) Ciaccio's Stain for Lipoids (Sections only)—(5) Accessory Methods for the Identification of Fats and Lipoids.
5. Identification of Glycogen: (1) General Notes—(2) The Langhans Iodine Method (Modified)—(3) Best's Carmine Method.
6. Identification of Mucin: (1) General Observations—(2) Fresh Preparations—(3) Sections: Methylene-Blue; Thionin; Mayer's Mucicarmine.
7. Identification of Pigments: General Notes—A. Artefact Pigments—B. Endogenous Pigments: (i) Iron-containing Pigments; (ii) Iron-free Pigments—C. Exogenous Pigments.
8. Identification of Yolk: (1) Examination of Bouin-fixed Material—(2) Distinction between Fat and Yolk.

INTRODUCTION

The microscopical identification of many cell and tissue components is largely morphological, being based rather on the size, shape, and distribution of such elements than on precise histo-chemical tests. Even morphological methods, however, often furnish some indication as to the chemical

nature of a cell component. For instance, imagine cytoplasmic granules stainable with iron-hæmatoxylin in paraffin sections after formol fixation. Their size, shape, and distribution suggest their being mitochondria. Fat would be excluded as a supposition since it is dissolved out of formol-fixed tissues during dehydration and clearing. If, however, the same tissue, fixed in Carnoy's fluid, but otherwise treated and stained in exactly the same manner, fails to show the intracellular granules, you would be justified in regarding these as being probably mitochondrial in nature, since the complex mixture of phospho-lipins constituting these bodies is dissolved in Carnoy's fluid, which is composed of powerful fat and lipoid solvents.

This example will at present suffice to show that even morphological methods, when considered in conjunction with those of fixation, permit of certain gross chemical inferences.

There exist, however, a certain number of methods, the use of which enables the investigator to "mark down" various organic or inorganic substances within the cell. The basis of such methods is usually due more to a definite colour reaction, invoked by combination of the substance under study and the reagents employed in its detection, than to a simple demonstration of its morphology.

Unfortunately the number of specific histo-chemical methods is at present very restricted. Still fewer of them are completely specific, *i.e.* they will sometimes stain compounds other than the ones they are specially destined to demonstrate.

Still, the fact remains that the intelligent and critical use of these histo-chemical methods is often of the greatest service.

New methods are at present constantly being invented, and it is to be hoped that the incursion of the Biochemis and his methods into the domain of minute morphology will be as fruitful in this as in the other departments of biological endeavour. It being largely the aim of this book to introduce reliable "type" methods to the worker, only one or two reactions are given for each substance.

Methods of purely pathological purport are not dealt with here but in the special chapter (*see p.* 303 *et seq.*).

Fresh Preparations.—By "fresh preparations" are meant dissociations of a tissue in normal saline, Ringer-Locke solution, or other media. Preparations of this kind are often used for histo-chemical tests.

MAKING A FRESH PREPARATION

To do this, remove a tiny fragment of the organ to be studied to a drop of normal saline or Ringer-Locke solution on a slide. Dissociate the tissue with a very fine needle held in each hand. A black slab (or piece of paper) between the slide and the table is helpful, while the best needles are very fine ones mounted in little wooden rods so that only 3 to 5 mm. of the needle projects. When dissociated, carefully let down a square ($\frac{7}{8} \times \frac{7}{8}$ inch) cover-slip to avoid bubbles. Such preparations, when critically examined with the microscope, are very valuable.

To apply histo-chemical tests to such preparations, add one or two drops of the reagent with a fine pipette to the left of the cover-slip; then induce a current of the reagent by placing a triangular piece of filter paper against the cover-slip on the opposite (right) side. With practice it is easy to make these manipulations without disturbing the slide as it lies on the stage of the microscope. For histo-chemical purposes, such observations are largely conducted with the 16 mm. ($\frac{2}{3}$ inch) or 4 mm. ($\frac{1}{6}$ inch) objective.

Avoid.—(i) Mounting in too large a drop of fluid, in which case the cover-slip and cells will move with the slightest vibration.

(ii) Allowing fluids, and especially acids, to come into contact with the objective. Should this occur, wipe it gently with cotton wool soaked in distilled water, then delicately dry it with a silk or linen rag.

(iii) Insufficient dissociation—the fault of the beginner. Lumps of tissue are not transparent.

The following list of histo-chemical methods for the identification of intracellular compounds is alphabetically arranged.

IDENTIFICATION OF ADRENALIN

Fresh Preparations

Adrenalin cannot be demonstrated.

Sections [1]

A useful technique devised by Cramer.

Fix thin slices of suprarenal gland at 37° C. to the vapour of 2 per cent. osmic acid for one and a half hours in the following manner. Take a small, wide-mouthed, glass-stoppered bottle. Put a segment of broad glass tubing, or another smaller bottle, inside the larger vessel. Place a piece of gauze on top of the tube (or small bottle). The pieces of tissue for fixation are placed on the gauze after removing surrounding fat, etc. In the case of the suprarenal of the mouse, the gland may be fixed *in toto*, and serial sections made of the entire organ.

Transfer the tissues to 60 per cent. alcohol, which is frequently renewed for twenty hours.

Dehydrate, clear (in xylol) and embed in paraffin in the usual manner.

Make thin sections of the tissues and mount on slides. Then treat as follows :

Slide 1.—Dissolve the paraffin with xylol, and mount direct in balsam or dammar. Both adrenalin and lipoid granules are stained black.

Slide 2.—Transfer from xylol to a tube of crude turpentine. Leave therein for twenty to thirty minutes. Rinse with xylol. Mount in balsam or dammar. All fats and lipoids having been dissolved out by the turpentine, only the adrenalin in the suprarenal medulla is stained.

Slide 3.—Treat with xylol, absolute alcohol, and 90 per cent. alcohol in the usual manner. Then place in a tube of 80 per cent. alcohol containing 10 per cent. of the commercial solution of hydrogen peroxide for thirty minutes. The brownish coloration of the tissues produced by the osmic acid

[1] I am greatly obliged to Dr. W. Cramer for supplying me with a detailed account of his technique.

will disappear. Then stain in Heidenhain's iron-hæmatoxylin. This slide will serve as a micro-anatomical control.

NOTE.—A careful removal of the fat surrounding the adrenal is essential before you fix the gland. If you attempt to fix through a dense layer of adipose tissue no results can be anticipated, since the osmic acid will undergo reduction in this and not in the adrenal gland.

2. IDENTIFICATION OF CALCIUM SALTS

(For *Bone*, see section on this in Special Methods, p. 209.)

Calcareous infiltration of tissues is normally restricted in the mammals to the special type of connective tissue known as bone. Hyaline cartilage also may show calcareous infiltration—especially in old age.

The distribution of calcium salts, of course, occurs throughout the tissues, but such calcium compounds are in a state of solution and extremely difficult, microscopically, to detect with any degree of precision.

Fresh Preparations

Calcium *deposits* in the tissues are soluble in acids. The following simple tests are applicable to smears or dissociations, but it must be remembered that they serve to demonstrate the presence of calcium compounds rather than their localisation :

(i) Add a very small drop of concentrated hydrochloric acid to one edge of the cover-slip. If calcium carbonate ($CaCO_3$) be present it will be dissolved, giving rise to bubbles of carbon dioxide.

(ii) If the above technique produces solution of the calcium deposit in the tissues but *no* effervescence, you are dealing with calcium phosphate.

(iii) Dissociate the tissues *not* in saline but in 40 per cent. alcohol. Add weak (*circa* 3 per cent.) sulphuric acid to a cover-slip slide preparation. If calcium salts be present, gypsum crystals ($CaSO_4$) will be formed.

NOTE.—This is an excellent test ; you dissociate in dilute alcohol because gypsum is very slightly soluble in water.

Methods for Sections

Treatment of material with acid solutions is, of course, counter-indicated both during and after fixation.

Avoid picric acid, as it slowly dissolves calcium salts. If you use formol as a fixative, make sure beforehand that it is neutral and does not contain free formic acid. If acid to litmus, neutralise by shaking with calcium carbonate or by adding concentrated caustic soda solution till neutral.

1. Von Kossa's Method

This technique depends on the formation of a precipitate of silver on deposits of lime salts.

1. Fix for a few hours in neutral 4 per cent. formol *or* in absolute alcohol.
2. Dehydrate, clear, and embed in paraffin.
3. Place sections on the slide for thirty to sixty minutes in a 1·5 per cent. aqueous solution of silver nitrate.
4. Rinse in distilled water.
5. Counter-stain the nuclei in safranin.
6. Dehydrate, clear, and mount in dammar or balsam.

Calcium salts appear as opaque-black areas in the section.

Notes.—(i) For rapid work the counter-stain can be omitted, and the sections mounted direct from stage 4 in dilute glycerine (pure glycerine and distilled water—equal parts). (ii) The distribution of minute deposits of lime salts is not as accurately shown as larger ones. This is because the silver deposit is deposited over and around the particles.

2. The Purpurin Method

1. Fix and section as above.
2. Bring sections on the slide into a saturated solution of purpurin in 96 per cent. or absolute alcohol for five to ten minutes.
3. Rinse in a 0·75 per cent. solution of sodium chloride for three to five minutes.
4. Differentiate in 70 per cent. alcohol until the stain ceases to pour out of the sections.

5. Dehydrate rapidly in absolute alcohol ; clear in origanum oil (xylol is as good) ; mount in dammar or balsam.

Calcium deposits are stained red.

3. IDENTIFICATION OF CHROMATIN

Although chromatin is a mixture of substances, it is usually possible to recognise it by its behaviour towards certain dyes, notably methyl-green.

The following tests may be applied :

(1) Fresh Preparations

1. Methyl-green-acetic acid. Dissociate the tissue in a drop of saline or Ringer-Locke solution on a slide. Cover. Run through a little concentrated solution of methyl-green in 1 per cent. acetic acid (formula, p. 111).

Chromatin is stained green. So also are mucin and silk fibres. Note that the " specificity " of methyl-green only holds good in the presence of acid or perfectly neutral solutions.

Since the acetic acid fixes the cells, the remarks made on p. 4 regarding the precipitation artefacts of chromatin must be borne in mind. Further, cytological observations on chromatin, especially in the resting nucleus, are incomplete without a study of the nuclei in the living cell.

(2) Enzyme Tests for Chromatin

As pointed out by Gatenby in the *Microtomist's Vade Mecum*, the application of such tests is a precarious business, and is beyond the reach of most workers. Further, such investigations certainly seem to need parallel control by standard biochemical methods of extraction.

(3) Sections

Here again methyl-green is the most reliable indicator for chromatin.

Fix preferably in mercuric chloride-acetic acid. Embed in paraffin. Stain sections on the slide in the pyronin-methyl-

green stain for ten to thirty minutes as described on p. 145. Differentiate (if necessary) by dipping the slide into tap water. Dehydrate rapidly in absolute alcohol.

Chromatin is fairly selectively stained green; so are chromatin nucleoli (karyosomes). Plasmosomes, red, likewise the cytoplasm of plasma cells and bacteria.

The methyl-green component of the stain fades after a few months.

4. IDENTIFICATION OF FAT AND LIPOIDS

(1) Value of Histo-Chemical Tests for Fat

The histo-chemistry of fats and lipoids is a very complex subject, still containing many lacunæ. Much of it is beyond the scope of a manual for the laboratory worker. Three reasons are largely responsible for the difficulty of identification of intracellular fat.

Firstly, the fat globules of the tissues are mixtures of fats, unsaturated and saturated. Consequently, such a reaction as intense blackening of globules with osmic acid is not an indication that these are unsaturated fats, but rather that the former are present in a greater proportion than saturated fatty compounds.

Secondly, many of the intracellular fatty and lipoidal compounds are "masked." That is to say, although they can be extracted and identified by standard chemical procedure, the application of appropriate stains, either to the fresh or sectioned tissues, will not reveal them. Thus, the cells of the renal tubules do not normally show fat on histo-chemical examination, though analysis reveals 15 to 20 per cent. of intracellular fat. Again, histological examination of normal heart muscle demonstrates only scanty fat globules, whereas in "fatty" degeneration similar study of the muscle cells reveals massive fatty changes. Yet chemical examination shows that the "fatty" heart contains *less* fat than the normal myocardium, the difference being that the normal heart muscle is especially rich in masked fats. The same holds good for fatty degeneration of the kidney. Masked

fats and lipoids are either in a condition of adsorption by, or of chemical combination with, the cell proteids, and, histochemical methods of identification not being able to break the association of the fatty substance—proteid combination, are mainly useless for the demonstration of such fatty compounds. Certain methods—*e.g.* that of Ciaccio—aim at rendering visible masked fatty compounds (in this case lipoids). It is probable, however, that the lipoids rendered visible by this method are adsorbed by the cell proteids rather than chemically combined with them (Policard).

Thirdly, the microscopical condition of intracellular fat appears largely to depend on the type of its colloidal distribution within the cell. Policard has shown that intracellular fat is present in the early stages of its formation as the continuous phase, the dispersed phase being formed by colloidal globules of proteids in solution. Fat is histologically invisible in this condition. But when the fat content of the tissues becomes greater than 25 per cent. (approximately), then a reversal of the phases occurs : the fat (previously continuous) becomes dispersed in the shape of fine droplets, which later fuse into globules, while the proteids in solution (previously dispersed) become the continuous phase. Only when the fat is dispersed can it be detected microscopically.

And, finally, it must be remembered that autolysed tissues may show fatty change—*i.e.* the fat becomes unmasked post-mortem.

These remarks are made to stress the following facts :

(1) That in a problem so complex, all that can be achieved in a general work on histological technique is to set forth a few reliable type methods for the identification of unmasked fats and lipoids.

(2) That purely histo-chemical research on fat and lipoids often needs biochemical control as an adjunct.

(3) *That fatty substances, when demonstrated microscopically, while furnishing trustworthy evidence of the amount of unmasked fatty substances present, do not reveal the masked—and often preponderating—fats and lipoids.* This point must be stressed, since many text-books on histological technique appear to ignore it.

Type Methods for the Identification of Fat

Certain considerations dominate the histo-chemical methods for the detection of fat.

1. Fresh (*i.e.* unfixed) fat globules are soluble in ether, chloroform, xylol, and high-grade alcohol—in brief, in the standard fat solvents. Consequently fixatives containing any of the substances (*e.g.* Carnoy's fluid) are to be avoided.

2. Intracellular fat is neither fixed nor dissolved by fixation in formalin. The tissues containing the fat are, of course, fixed, but the fat globules themselves are untouched. This means that while such fat may be stained with appropriate fat stains, it will be immediately dissolved by high-grade alcohol, xylol, etc.

3. Intracellular fat, after treatment with osmic acid, is rendered sufficiently insoluble to resist, under appropriate conditions, the action of many fat solvents.

(2) The Scharlach R and Sudan III. Methods

The principle underlying this stain (and also Sudan III.) is that the stain is more soluble in fat than in the medium it is dissolved in. Staining of fat by it is therefore a purely physical process.

Fresh Preparations

1. Dissociate the tissue in the minimum of fluid on a slide.

2. Fix in vapour of concentrated (40 per cent.) formol for thirty to sixty seconds.

3. Treat with 50 per cent. alcohol for three to five minutes.

4. Place the slide in a Petri dish containing a saturated solution of Scharlach R in equal parts of 70 per cent. alcohol and acetone. This stain should be filtered and kept in a well-stoppered bottle; evaporation during the staining process must be avoided. If these precautions are not taken, precipitates will form. Stain thirty to sixty minutes.

5. Rinse in 50 per cent. alcohol.

6. Mount in dilute glycerine or glycerine jelly (formula, p. 119).

Fat globules are stained red.

Sudan III. (a saturated solution in 70 per cent. alcohol) is used in the same way.

NOTE.—After stage 5 you can counter-stain the nuclei in Delafield's hæmatoxylin in the usual manner. Avoid high-grade alcohol, and all other fat solvents throughout the process.

The application of Scharlach R to fresh preparations is especially useful for the rapid identification of fat.

Scharlach R is a permanent stain, but the hæmatoxylin will fade fairly soon, owing to its solubility in the water of the mounting media.

SECTIONS

1. Fix small pieces of tissue—not thicker than 3 mm.—in 5 per cent. formol for three to twenty-four hours.
2. Wash in running water for a few hours.
3. Embed in gelatine by the method detailed on p. 87; *or* soak the tissues in a weak aqueous solution of gum arabic for a few hours;
or place direct from 2 on the microtome.
4. Cut frozen sections.
5. Collect these in a dish of 50 per cent. alcohol.
6. Stain in Scharlach R or Sudan III.
7. Rinse in 50 per cent. alcohol.
8. Counter-stain the nuclei in Delafield's hæmatoxylin . . .
9. Blue the hæmatoxylin . . .
10. Rinse in 50 per cent. alcohol . } Exactly as for fresh preparations.
11. Mount in glycerine or (better) glycerine jelly . . . .

Results as above.

NOTES.—(1) Scharlach R is very powerful and can be applied even to material which has been preserved in formol for months.

(2) True fats are intensely stained: cholesterinesters, and cholesterin—fatty acid mixtures less intensely. Other lipoids do not (generally) stain.

(3) Methods of Osmication

The blackening of fats by osmic acid is less precise a reaction than the above, since the various lipoids are also browned

by it. The fact, however, that lipoid granules are small, while fat globules—except in the early stages of their formation—are comparatively large, enables one to distinguish between them in most instances.

The blackening of fat by osmic acid is due to reduction of the OsO_4 to a lower hydrated oxide—probably having the formula $OsO_2.5H_2O$ (Huntingford and Partington).

Fresh Preparations

Either expose the dissociated tissues to the vapour of 2 per cent. osmic acid for ten minutes, *or* run a drop of this solution beneath the cover-slip. Fat will turn black or dark grey.

Sections

1. Fix thin pieces of tissue in Flemming's fluid for twenty-four to forty-eight hours. If you employ the ordinary variety plus acetic acid, the mitochondria will be more or less distorted and destroyed. If you use Gatenby's modification (from which the acid is omitted) the mitochondria will be preserved.
2. Wash in running water for twelve hours.
3. Proceed through upgraded alcohols.
4. Clear rapidly in xylol or benzol (*i.e.* thirty minutes).
5. Embed rapidly in paraffin (*i.e.* thirty to forty-five minutes).
6. Make sections on the slide.
7. Dissolve the paraffin with xylol.
8. Mount in xylol balsam or dammar.

Fat globules, black to dark grey; lipoidal granules, brownish.

Notes.—(i) Osmicated fat is only relatively insoluble in clearing reagents. Therefore treat for the minimum time with these reagents consistent with proper de-alcoholisation.

(ii) After dissolving the paraffin from the sections, you can stain with safranin, or with any other powerful dye, with advantage.

(iii) Yolk discs or granules may stain black with osmic acid if they contain much fat. For the distinction between yolk and fat, see p. 184.

(4) Ciaccio's Stain for Lipoids

This method gives a positive stain for lipoidal granules, while fat is dissolved out of the cells, and hence not shown. It is only applicable to sections.

The principle underlying this method is that fat is not rendered insoluble by potassium bichromate (except when this substance is associated with osmic acid). Lipoids, on the other hand, are preserved, and mordanted, by potassium bichromate.

1. Fix thin pieces of tissue for forty-eight hours in the following fluid :

5 per cent. potassium bichromate	80 c.c.
40 per cent. (*i.e.* concentrated) formol	20 c.c.
Glacial acetic acid	5 c.c.
or	
Formic acid	4 to 5 drops.

2. Post-chromatisation in 3 per cent. potassium bichromate for five days (generally twenty-four to forty-eight hours is ample treatment).

3. Wash in running water for twenty-four hours.

4. Dehydrate in ascending grades of alcohol. Reduce the duration of the 90 per cent. and absolute alcohol baths as much as possible.

5. Xylol—absolute : one hour.

6. Pure xylol : one hour.

7. Xylol-paraffin : one to one and a half hours.

8. Pure paraffin : one to one and a half hours.

9. Make sections on the slide.

10. Dissolve the paraffin with xylol.

11. Pass from absolute alcohol into :

12. A saturated solution of Sudan III. in 95 c.c. of 85 per cent. alcohol plus 5 c.c. of acetone. Stain in a stoppered slide jar for thirty to sixty minutes at (approximately) 30° C.

13. Dip the slide very rapidly (one second) in 50 per cent. alcohol to differentiate the stain.

14. Rinse in water.

15. Counter-stain in Delafield's or Heidenhain's hæmatoxylin (optional).

16. Rinse in water and blue the hæmatoxylin (in the case of Delafield).

17. Mount in Apathy's medium (formula, p. 119). Glycerine jelly may also be used.

The lipoidal substances appear as fine orange granules. Nuclei, blue to black, according to the type of hæmatoxylin employed.

(5) Accessory Methods for the Rapid Identification of Fats and Lipoids

1. Both fats and lipoidal granules appear as refringent globules in fresh preparations.

2. Examination of a fresh preparation in polarised light. Fats and fatty acids do not show double refraction in polarised light. With crossed Nicols they are invisible. Lipoids are doubly refractive and appear, with crossed Nicols, as luminous granules. Further, on gently heating the slide to *circa* 60° C. the double refraction disappears, but returns on cooling. In frozen sections of formol-fixed material the lipoids behave typically in polarised light, but are often needle-shaped (Cramer).

The fact that fats and fatty acids are isotropic, and lipoids anisotropic, constitutes a useful differential test for fresh material—provided one possesses the necessary polariser and analyser.

3. The presence of large empty vacuoles in cells fixed by all fixatives —*except* those containing osmic acid—after embedding in paraffin points, nearly always, to intracellular fat dissolved during dehydration and clearing.

5. IDENTIFICATION OF GLYCOGEN

(1) General Notes

Fresh Preparations.—Glycogen cannot reliably be detected.

The distribution of glycogen has already been summarised. But once again the fact may be stressed that the fine granules, or the amorphous masses, of glycogen seen in fixed and stained specimens probably do not represent the actual state in which this substance exists in the living cell. Intravitam glycogen is in a state of solution, and it is only the precipitated product which is microscopically discernible.

Material for the demonstration of glycogen should be as fresh as possible, since this product may become transformed into sugar after death. But it has been noted that glycogen may persist microscopically unchanged for as long as twelve hours after death. After this a progressive decrease has been observed (Takenuchi).

Remember, as a general rule, when wishing to detect glycogen, that it is soluble in water and in aqueous media in general. Therefore avoid rinsing pieces of tissue in normal saline before fixing them.

There exist two methods which are especially useful for staining glycogen : the iodine technique (which is the easier and surer in action), and Best's carmine method (which gives more definite pictures of intracellular glycogen but is more awkward to work with).

(2) The Langhans Iodine Method (Modified)

1. Fix thin slices of tissue in absolute alcohol.
2. Clear in xylol.
3. Embed in paraffin.
4. Mount sections on the slide, using in place of the solution of glycerine and albumen in water the same dissolved in 50 per cent. alcohol.
5. Treat with xylol and absolute alcohol.
6. Stain sections in Lugol's solution (formula, p. 117) for five to ten minutes. Pour off and wipe dry around the sections.
7. Dehydrate in a saturated solution of iodine in absolute alcohol. Wipe rapidly.
8. Clear and differentiate in oil of origanum.
9. Wash in xylol to arrest differentiation.
10. Mount in dammar or balsam.

NOTES.—(1) Fixation in Carnoy's fluid (formula, p. 39) is optional. It produces less shrinkage than pure absolute alcohol.

(2) You can counter-stain in Delafield's hæmatoxylin after stage 5 (differentiating *not* in dilute hydrochloric acid, but in acid alcohol).

(3) The standard technique is to mount either in origanum oil (and then ring the cover-slip) or in origanum oil-balsam. This is

illogical, since origanum oil differentiates the iodine stain. Consequently such sections fade very rapidly.

(4) No matter how the sections be mounted, the stain tends to fade. Its permanency is usually less than one year.

Glycogen is stained brownish red. The nuclei, if stained in hæmatoxylin, are greenish.

(3) Best's Carmine Method

Fix as for the iodine technique.

Embed either in paraffin or in celloidin.

If paraffin sections are employed, they must, after fixation to the slide by the modified glycerine and albumen method, be placed overnight in a 1 per cent. solution of celloidin in absolute alcohol. They are then drained, allowed to dry, and treated like celloidin sections—*i.e.* absolute alcohol is avoided.

In either case you stain as follows :

1. Bring the sections down to water.
2. Stain in Delafield's or Ehrlich's hæmatoxylin. (If you differentiate, use acid alcohol.)
3. Blue in ordinary tap water.
4. Stain in Best's carmine solution (formula, p. 108) for five minutes.
5. Differentiate for one to five minutes in Best's differentiator (formula, p. 109). When no more carmine comes away from the tissues :
6. Dehydrate in 90 per cent. alcohol and xylol-acetone (p. 147).
7. Clear in xylol.
8. Mount in dammar or balsam.

Glycogen, red ; nuclei, blue. The stain is permanent.

Notes.—(1) Remember that the carmine solution, once prepared, is unstable, especially in summer.

(2) The best results are given by celloidin-embedded material.

(3) For research purposes it is necessary to integrate the results furnished by both iodine and carmine methods. As a further precaution, to guard against the staining of granules other than glycogen by Best's carmine, a section is spat upon and set aside for ten minutes. The

diastase in the saliva dissolves the glycogen. This section is then rinsed and stained with the carmine solution. No red granules should be present in it.

(4) Best's method is said to be not as specific as the Langhans iodine technique, but it has the great advantage of not fading.

6. IDENTIFICATION OF MUCIN

(1) General Observations

The mucin of the histologist is an inter-cellular secretory product formed especially in various epithelia.

Biochemically there are many mucins of slightly different chemical composition: the term mucin (though convenient) does not, it must be remembered, refer to a single chemical entity such as adrenalin.

Histo-chemically, mucin has the following characteristics:

(*a*) Great affinity for the basic dyes in general. This is due to the acid reaction of mucin.

(*b*) Metachromatic staining with certain basic aniline dyes (for a definition of metachromasy, see p. 97).

(*c*) Solubility in weak alkalies.

(*d*) Precipitation by acetic acid.

(2) Fresh Preparations

These yield tests (*a*) to (*c*). Test (*d*) is more difficult to see. The staining reactions of mucin, being the same in fresh preparations as in sections, are described beneath.

(3) Sections

The tests for mucin are applicable after most fixatives, but the best results are obtained after fixation in a saturated aqueous solution of mercuric chloride. Corrosive sublimate-formol also gives good results. Embed in paraffin in the usual manner.

1. Methylene-Blue Method.—Stain sections on the slide in Borrel's methylene-blue (formula, p. 110) diluted in the proportion of one part of stain to five of distilled water for two to five minutes. Differentiate in 90 per cent. alcohol,

controlling the process with the staining microscope. Clear in xylol. Mount in dammar or balsam.

2. Hoyer's Thionin Method.—Stain sections for five to fifteen minutes in the following solution :

A saturated aqueous solution of thionin	2 drops.
Distilled water	5 c.c.

Dehydrate rapidly. Clear in xylol. Mount in dammar or balsam.

Mucin by both the above methods is metachromatically stained *purple*, the other tissue constituents being in shades of blue.

Note, however, that the ground substance of cartilage, the basophil granules of mast cells, and Wharton's jelly of the umbilical cord stain also purple. This is because of their similar chemical composition to mucin.

3. Mayer's Mucicarmine Method.—This technique gives a specific staining reaction for mucin.

Stain sections thus :

1. Dilute one volume of stock mucicarmine solution (formula, p. 109) with ten volumes of tap water. The more alkaline the latter, the better. Stain five to ten minutes.

2. Rinse in tap water.

3. Dehydrate, clear, and mount as usual.

Mucin is stained red. You can counter-stain the nuclei in Delafield's hæmatoxylin before treating with mucicarmine.

Notes.—(i) The stock solution keeps indefinitely ; the diluted solution does not. Therefore prepare the latter immediately before staining.

(ii) Should the nuclei be at all stained, this is because the solution contained free acid, in which case it should be carefully neutralised by the addition of a saturated aqueous solution of sodium bicarbonate.

(iii) Usually the metachromatic staining of mucin with methylene-blue or thionin suffices for its identification. If the reaction is in any way obscure, supplement this by the mucicarmine test. Remember that safranin usually stains mucin metachromatically. In such cases mucin is yellow ; so also are mast-cell granules and the ground substance of hyaline cartilage.

7. IDENTIFICATION OF PIGMENTS

General Notes

The substances known as "Pigments," mostly found as intracellular granules, form a heterogeneous group, many of the members of which have no chemical affinities. For, as pointed out by Adami and M'Crae, "Possession of colour is no adequate ground of relationship"—except, perhaps, as a convenient temporary measure. Very little is known regarding the physiology and mode of formation of animal pigments. Moreover, only the gross bearing of the composition of pigments on histochemical methods for detecting them can be considered here.

The pigments encountered in tissues may be roughly divided into three main groups according to their origin. Thus, there are (1) the "*Artefact*" *pigments*. The black, granular precipitate of mercuric chloride in improperly treated material, and the precipitate sometimes caused by formol fixation, come under this heading; (2) the *Endogenous pigments*, produced by the cell's metabolism, *e.g.* the pigment of the skin—Melanin; (3) the *Exogenous pigments*, derived from without the organism, such as the various dusts (*e.g.* carbon) inhaled into the lungs.

A. Artefact Pigments

It must be remembered that when tissues which have undergone post-mortem changes are fixed in formol, a fine brown to black deposit is often encountered in sections. Material derived from the post-mortem room frequently shows this precipitate, which is most profuse inside and around blood vessels, and in organs containing much blood. This pigment is derived from hæmoglobin which has been broken down by post-mortem changes—whence its profusion among red blood corpuscles. Its histo-chemical reactions approximate towards those of the melanins.

It may be dissolved by treatment of the sections with 1 *per cent. or* 2 *per cent. caustic soda or potash in* 80 *per cent. alcohol.*

Distinction between the artefact pigments and those present in living cells and tissues may be made by bearing the following considerations in mind:

1. The pigment can be seen to lie on top of the cells rather than inside them (on careful focusing). It may also be found in the spaces between the tissues.

2. Its presence is dependent on a definite technique of fixation, staining, or impregnation. Thus, the mercuric chloride precipitate is only found after treatment of tissues with this substance, and is soluble in iodine. The formol-produced precipitate is only found in organs which have been fixed in this substance some time after death. Again, the precipitate often formed during impregnation with silver nitrate is only found after such treatment, and is especially abundant at the periphery of the tissues. As for precipitates of stains—always due to faulty technique—these are usually highly coloured in the sections.

B. Endogenous Pigments

The following tests are applicable both to fresh preparations and to sections; but the most accurate results are obtained with the latter.

(i) Iron-containing Pigments

Apart from various solubility tests, the most important reactions for the detection of the iron-containing pigments are based on the chemical interaction of the iron with ammonium sulphide $[(NH_4)_2S]$ to form black iron sulphide FeS, or with potassium ferricyanide $[K_3(Fe(CN)_6)]$ to form Turnbull's blue (ferrous ferricyanide, $Fe_3(Fe(CN)_6)_2$, or with potassium ferrocyanide $[K_4(Fe(CN)_6)]$ to form Prussian blue (ferric ferrocyanide, $Fe_4(Fe(CN)_6)_3$).

Note.—*Iron in tissues is usually combined with proteid matter, and it is hence necessary to free or unmask it from this by treatment with a mineral acid before it will give the iron reactions mentioned above. Sometimes the iron is so intimately combined with the proteid molecule—as in hæmoglobin—that it is impossible histo-chemically to free it. Most of the iron-containing pigments, however, easily give up their iron.*

Hæmosiderin

This pigment is hydrated ferrous oxide combined with proteids. It is one of the hæmoglobin derivatives, being one of the products formed by the breaking down of this sub-

stance. It is rarely demonstrable in the normal organism, though abundant under certain pathological conditions.

Occurs as golden or brown particles.

Reactions of Hæmosiderin and Other Iron-containing Pigments

Soluble in acids; insoluble in alkalis and fat solvents (ether and chloroform); cannot be bleached; not blackened by treatment with osmic acid or silver nitrate; give the following special reactions for iron (best applied to sections).

Fixation of Tissues

Fix tissues in absolute alcohol. Formol or mercuric chloride fixation of short duration may also be used, but alcohol is to be preferred. Avoid the use, both during and after fixation, of solutions containing iron or acids. Avoid also fluids containing potassium bichromate. The distilled water used for making up reagents must be iron-free, since traces of iron in such fluids will combine with the calcium salts in the tissues and then give an iron reaction.

Make frozen, paraffin, or celloidin sections with a rust-free knife.

Test 1.—*Prussian Blue Reaction.*—The iron in the tissues combines with potassium ferrocyanide [$K_4Fe(CN)_6$] as follows:

$$4FeCl_3 + 3K_4Fe(CN)_6 = Fe_4(Fe(CN)_6)_3 + 12KCl.$$

The blue precipitate thus formed is the Prussian blue of the chemist. The HCl is used to liberate the masked iron.

Fix and section the material as above.

1. Rinse the sections in distilled water.
2. Put them in the following freshly made-up solution:

2 per cent. aqueous solution of potassium ferrocyanide	1 part.
1 per cent. hydrochloric acid	3 parts.

Leave therein for twenty to thirty minutes. If it is necessary to prolong the staining, the solution should be renewed from time to time.

3. Transfer to distilled water.
4. Counter-stain in eosin or safranin (optional).

5. Mount in glycerine jelly or dehydrate, clear, and mount in dammar or balsam in the ordinary way.

Ferric salts are stained blue. The iron salts in tissues are usually ferric.

TEST 2.—*Turnbull's Blue Reaction.*—The basis of this is the formation of a blue precipitate ("Turnbull's blue") due to the following reaction :

$$3FeCl_2 + 2K_3Fe(CN)_6 = Fe_3(Fe(CN)_6)_2 + 6KCl.$$

(Ferrous ferricyanide.)

It is far less useful than Test 1, since it only shows ferrous salts, which are less common than ferric ones.

Fix and section as above.

1. Rinse the sections in distilled water.

2. Treat in the following freshly prepared mixture for five to fifteen minutes :

2 per cent. aqueous solution of potassium ferricyanide .	1 part.
2 per cent. hydrochloric acid	3 parts.

3. Transfer to 1 per cent. hydrochloric acid for five to fifteen minutes.

4. Rinse in distilled water.

Counter-stain (if desired) and mount as for Method 1.

Ferrous salts are stained blue.

TEST 3.—*Ammonium Sulphide Reaction.*—This method stains both ferric and ferrous salts, owing to the formation of ferrous sulphide (FeS).

In the case of ferr*ous* salts the reaction is as follows :

$$(NH_4)_2S + FeCl_2 = FeS + 2(NH_4)Cl.$$

In the case of ferr*ic* salts the reaction proceeds in two almost simultaneous stages, in the first of which the hydroxyl ions of the ammonia cause the precipitation of gelatinous ferric hydroxide, which is then transformed into ferrous sulphide by the reducing action of the hydrogen sulphide. The equations are :

1. $FeCl_3 + 3NH_4OH = Fe(OH)_3 + 3NH_4Cl.$

(Ferric hydroxide)

2. $2Fe(OH)_3 + 3H_2S = 2FeS + 6H_2O + S.$

(Ferrous sulphide)

Fix and section as above.

1. Rinse the sections in distilled water.

2. Put them in a concentrated yellow solution of ammonium sulphide $(NH_4)_2S$ for five minutes to half an hour. The sections are ready when they are dark green or blackish in colour. The best results are given by an old and yellow solution of ammonium sulphide.

3. Wash rapidly in distilled water.

Counter-stain (if desired) and mount as for Method 1, but use (preferably) origanum oil balsam instead of xylol balsam or dammar.

Iron salts (ferric and ferrous) appear as green or dark green granules.

NOTES.—For ordinary purposes, Test 1 is to be recommended. Test 3 is useful in that it enables both ferric and ferrous compounds to be stained. But it is NOT specific for iron, since *salts of silver, mercury, and lead are also stained by this method.*

Hæmoglobin.—The iron of this compound cannot be unmasked—not even after treatment for one year at 55° C. with ammonium hydrogen sulphide (Mann). The above tests are consequently useless for the detection of hæmoglobin. The normal association of hæmoglobin with the red blood corpuscle and its bright red staining with Mann's methyl-blue-eosin (p. 134) suffice for its detection with the microscope.

(ii) Iron-free Pigments

So much uncertainty still prevails regarding the microchemical tests for these substances that only two of the most important groups are dealt with here.

(i) MELANIN.—Normally found in the skin, choroid of the eye, and in the brain.

Occurs as granules or needles.

REACTIONS.—Insoluble in acids and fat solvents; soluble in concentrated alkalies; gives no iron test; bleached by chlorine and hydrogen peroxide; unaffected by fat and lipoid stains; blackened by silver nitrate and osmic acid.

The above tests are best applied to fresh preparations.

NOTE.—Melanin is sometimes associated with coloured lipoids. In such cases the melanin may be bleached by treatment with dilute

hydrogen peroxide, and the lipoidal components then stained with a 1 per cent. aqueous solution of neutral red.

(ii) Fatty Pigments.—Lutein, the bright yellow pigment normally present in the corpora lutea, is an example of these. They may be recognised by their behaviour towards fat solvents and fat stains.

Fatty pigments are dissolved in fat solvents (ether and chloroform); blackened or browned by osmic acid; not blackened by silver nitrate.

Stained red with Scharlach R;
Stained yellow with Sudan III. } For technique, see p. 169.

The above tests are best performed on fresh preparations.

C. Exogenous Pigments

Of these the most important are particles of coal, soot, and other forms of carbon inhaled into the lungs. Such particles are very largely intracellular. To distinguish between iron pigments, melanin, and carbon particles, treat fresh preparations or sections as follows:

Treat with concentrated sulphuric acid: if the pigment resists this, it is either carbon or melanin.

Treat with hydrogen peroxide: if the pigment is bleached, it is melanin. If unaffected: carbon.

8. IDENTIFICATION OF YOLK

As already pointed out (p. 19) yolk is a mixture of many substances. Consequently, there exists no histo-chemical test for yolk, since the type of reaction furnished is governed largely by the substance present in the greatest proportion. Roughly speaking, this is either fat or proteids.

The appearance of yolk in usually fair-sized granules or discs, its yellowish, refringent character in fresh preparations, and its restriction to the egg-cells, or, in vertebrates other than mammals, to embryonic tissues, usually render its identification fairly easy on purely morphological grounds.

The application of the two following methods enables the distinction to be made between yolk and true fat globules.

(1) Examination of Bouin-fixed Material

Fix material in Bouin's fluid, 4 per cent. formol, or mercuric chloride. Dehydrate, clear, and embed in paraffin. Stain sections on the slide with any simple staining method—*e.g.* Delafield's hæmatoxylin and eosin.

Fat globules are dissolved out, so likewise the fatty components of yolk granules, but the proteid basis persists, and can be demonstrated as a finely granular, vacuolated mass, usually oxyphil in reaction.

(2) Distinction between Fat and Yolk

Fix in Champy's or Flemming's fluids. Dehydrate, clear, and embed. Reduce the xylol and paraffin baths to the minimum. Treat the sections with xylol as usual, then place them in a tube of turpentine. Leave therein for one to three hours. Both fat and yolk are decolorised in this medium, but, in the case of yolk, the proteid components of the yolk granules can be subsequently stained with Heidenhain's hæmatoxylin, whereas the globules of pure fat leave only empty vacuoles.

CHAPTER XII

INJECTION METHODS

1. Introduction—2. Apparatus—3. Preparation of an Injection Mass—4. Technique of Injection—5. Methods for Injecting Capillaries

1. INTRODUCTION

The injection of a coloured and insoluble substance into the blood vessels or lymphatics was more often resorted to in the past than nowadays. But for certain work the method is still useful.

There exist two methods of injection—the "cold" and the "warm." The latter is the better for most purposes. The principle of the warm method is the distension of blood vessels, etc., with a solution of warm gelatine. On cooling, the gelatine solidifies. To allow of the tracing of the path taken by the injection mass, some insoluble dye, usually carmine or Prussian blue, is added to the gelatine. For making satisfactory injections the mass must be colloidal: if the particles are too large, the capillaries will not be injected, while if the mass is in a state of true solution, it is liable to diffuse through the vessel walls. The necessity for a colloidal mass renders the preparation rather tiresome. Injection masses may be bought ready made up from Grübler.

2. APPARATUS

(*a*) *A large syringe.*—This is only suitable for the injection of individual organs—for instance, a spleen or a kidney of a medium-sized mammal. A segment of indiarubber tubing and a clip are interposed between the nozzle of the syringe and the cannula.

(*b*) *A funnel, the height of which is adjustable, connected*

to the cannula by a length of indiarubber tubing.—This is not as good as (*c*) for the injection of warm masses, as the funnel cools rather quickly. But for cold masses it is simple

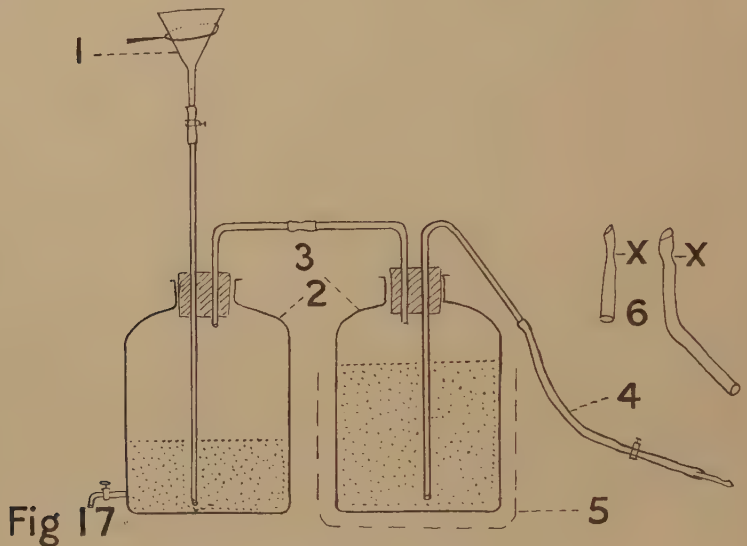


FIG. 17.—INJECTION APPARATUS OF TOLDT.

1. Funnel fitted to a tall glass tube. The length of this need not be more than 15 cm., since this will give a pressure of over 2 metres of water, which is more than enough for all injection work.

2. Mercury flask supplying the air pressure for:

3. Flask containing the injection mass.

4. Tubing connecting the contents of 3 with the cannula.

5. Water bath. For gelatine injection masses this is filled with hot water to melt the mass. The temperature may be kept up by a small flame beneath the water bath.

6. Cannulæ for injection. Note the neck (*x*) on these; this constriction is essential in order that the cannula may be securely tied into the vessel or duct.

A screw clamp should be interposed beneath the mercury funnel and another near the cannula. The injection pressure is regulated by varying the height of the mercury funnel; the rate of outflow of the injection mass is controlled by the screw clamps.

and very good. Since it is necessary to wash out the blood before passing in the injection mass, two such funnels, connected to a Y tube close to the cannula, are requisite. One is filled with warm saline, the other with the injection mass.

(c) *A constant pressure apparatus in which the bottle containing the injection mass is placed in a water bath.*—This is the most suitable device for warm injections.

Fig. 17 illustrates this type of injection apparatus.

3. PREPARATION OF THE INJECTION MASS

A. **Carmine-Gelatine** (*for warm injections only*).—Prepare the following solutions :

SOLUTION A

Gelatine. .	250 grms.	Dissolve on the water bath
Distilled water .	750 c.c.	at 60° C.

SOLUTION B

Carmine rubr. opt. .	150 grms.
Distilled water .	75 c.c.
Ammonia . .	25 c.c.

Add B to A, and stir thoroughly. Then cautiously add 50 per cent. acetic acid, about 1 c.c. at a time. Stir thoroughly after each addition of the acid. The mass will change from the transparent to the opaque, and the smell of ammonia nearly or completely disappears. *Immediately this occurs stop adding the acid,* as the neutral point has been reached.

The neutralisation of the mass is rather delicate. If the carmine gelatine is acid, it will form lumps in the vessels ; and if alkaline, it will diffuse through the vessel walls.

Finally, filter the hot mass through flannel into a 1-litre bottle to which 2 grammes of crystallised phenol has been added.

B. **Ranvier's Prussian-Blue** (*when not mixed with gelatine, a cold injection mass*).—Make a saturated solution of ferric sulphate in distilled water. Add this gradually to a saturated aqueous solution of yellow prussiate of potash (potassium ferrocyanide). Wash the precipitate (=Prussian blue) on a felt strainer beneath which a glass funnel and filter paper have been arranged. After a few days the fluid from the second filter will be blue. Now turn out the contents of the felt strainer into distilled water. They will slowly

dissolve. The solution is now ready for injection. *When mixed, after warming, with 4 per cent. of gelatine, it can be used for warm injections.*

4. TECHNIQUE OF INJECTION

The best and most reliable injections are those made immediately after death. If there be rigor mortis, the injection of muscle is not easy and must be prolonged.

Individual organs are injected by the main artery either *in situ* or after removal. Thus, the kidney is injected through the renal artery. The renal vein is severed to allow the blood to be washed out prior to injection.

To inject the head region the carotid is usually taken. For a total injection the aorta is satisfactory. The cannula should be securely tied in, and air-bubbles are to be avoided, by seeing that both cannula and tubing are full of fluid. *A small amount of air will ruin an injection, since it is usually impossible to expel it from the vessels in which it causes veritable "air emboli."*

Supposing that you are going to inject the blood vessels of the brain, the procedure may be outlined as follows :

1. Rapidly expose a carotid in an animal which has just been killed with chloroform.
2. Pass a loop of ligature round the vessel at the point where you intend to insert the cannula ; make the first noose of a reef knot, but do not tighten it.
3. Make a small transverse incision in the artery with a pair of very fine scissors.
4. Take a cannula out of a dish of saline and insert it so that its aperture in the artery faces headwards.
5. Tighten the loop and tie the cannula in firmly. See that the latter is full of saline.
6. Open a large vein (*e.g.* the jugular).
7. Proceed with the injection, running in firstly saline, then the injection mass. Do not perfuse with the latter until the saline is not at all—or only slightly—discoloured with blood as it issues from the sectioned vein.

The organ or animal is first washed out with warm (38° to 40° C.) isotonic saline. For a whole cat some 250 to 500 c.c.

are necessary. A water pressure of about 1 metre (*i.e.* 7 to 8 cm. Hg) is usually sufficient.

Next, the injection mass is passed through the cannula. The pressure may now advantageously be raised to $1\frac{1}{2}$ metres (*i.e.* 10 to 11 cm. Hg).

When the tissues are evenly injected, the blood vessels supplying the organs to be studied are ligatured, and the tissues are fixed in 96 per cent. alcohol. Or you may employ formol. The colder the fixative, the better.

NOTE.—Often a better injection is given if the vein or veins are tied off at the end of the injection, but the pressure kept up for a minute or two before ligaturing the artery.

Since both animal and injection mass are losing heat, speed in all warm injections is important. For work on the cold cadaver, either employ the Prussian blue technique or place it in a bath of warm saline and inject it in this with carmine gelatine.

Should any leakage of the mass occur from tissues during injection, clamp the leaking areas with artery forceps. Such mishaps are usually due to trauma during exposure of the vessels and tissues.

5. METHODS FOR INJECTING CAPILLARIES

Sometimes it is difficult to get the gelatine injection into capillaries. Fischer's method is useful in such cases :

1. Wash out the blood with sodium sulphate or sodium nitrate of 8 per cent.
2. Inject milk.
3. Fix the organs, after ligature of their blood vessels, in the following fluid for at least twenty-four hours :

40 per cent. formol . . .	75 c.c.
Glacial acetic acid . . .	15 c.c.
Water	1000 c.c.

4. Place slices of the tissue in water to remove the formol and acetic acid.
5. Make frozen sections.
6. Apply a standard fat stain, such as Scharlach R or

Sudan III. (see p. 170) followed by hæmatoxylin. Mount in glycerine or glycerine jelly.

The fat globules of the milk are stained red.

As a substitute for the milk, you can inject 100 grammes of pig's fat [*i.e.* lard] dissolved in 500 c.c. of ether (Schmorl). Fix and stain as above, but wash very thoroughly in water to remove the ether. If you fail to do this, the tissues will be very difficult to freeze.

CHAPTER XIII

METHODS OF VITAL STAINING

1. Introduction—2. Technique of Vital Staining: (i) Methylene-Blue; (ii) Neutral Red; (iii) Janus Green; (iv) Isamine (=Pyrrol) Blue; (v) Trypan Blue.

The technique of staining living cells, either by injection of the whole organism or by bathing the cells in the staining solution, received its first impetus from Paul Ehrlich in 1885.

1. INTRODUCTION

Certain features of this valuable method must be stressed.

1. The comprehensive title of vital staining only indicates that certain cytoplasmic elements may be stained in cells without any serious or obvious impairment of their vitality. It seems clear, for instance, that the nucleus has never yet been stained in the living cell. Permeability of the nuclear membrane towards dyes appears to be an indication of the death of the cell. Not so, however, with the surface membrane of the cell which, under certain conditions, behaves as a permeable membrane towards certain dyes. The elements which stain intravitally appear all to be cytoplasmic—mitochondria, vacuoles, and various metaplasmic inclusions. The ground substance does not stain in the living cell. It is only when the cell has died, or been seriously injured, that the dye spreads to the other cytoplasmic and nuclear elements.

2. All vital dyes are toxic if used in a sufficiently high concentration. Whence the need for keeping the latter as low as is consistent with the staining of the elements you wish to study.

3. The images furnished by the preservation of vital dyes in *fixed* specimens must be approached critically. The up-

heaval produced by fixation in the colloidal system of a living cell has already been mentioned (p. 29), and enough has been said to make clear the need for closely comparing the appearances seen in the living stained or unstained tissues with those furnished by the "fixed" vital dye, before coming to any conclusion as to the validity of the images furnished by the latter. Furthermore, such appearances are only "vital" in the sense that a living cell *had* been stained.

4. The illuminant used for work with intravitam dyes is very important. Not only should the colour of the illuminant be one which does not mask the vital staining, but its intensity should be constant. This means that daylight, which varies both in colour and intensity, is unsuitable. An opal electric bulb, or an adequately dimmed Pointolite, is to be preferred. A "cold" light with a tinge of blue is usually the best for observation, and the least trying to the eyes. A blue glass screen, or a piece of "daylight glass," will give such a light.

5. In the case of non-motile cells, such as comprise the majority of those of mammals, it is difficult to say whether such cells are alive, moribund, or actually dead. Much of the most reliable work on intravitam staining has therefore been obtained from the study of tissue cultures, where the phenomena of invasion of the medium and of cell division furnish definite criteria as to the vitality of the cells under observation (M. R. and W. H. Lewis, and others).

6. Certain of the vital dyes, of which methylene-blue and Janus green are examples, have a very curious property. The presence of oxygen is essential for staining to take place. Thus, tissues which have been stained intravitam with methylene-blue lose their colour after a short time, when mounted in an indifferent medium (serum or normal saline) between slide and cover-slip. This was shown by Ehrlich to be due to the conversion of the methylene-blue into a colourless compound, its *leuco-base*. Re-exposure of the tissues to oxygen (*e.g.* by removal of the cover-slip) causes the leuco-base to be oxidised into the dye-stuff once again. If the tissues be kept in a confined space, their avidity for oxygen rapidly reduces the dye. A similar phenomenon has been noted in regard to vital staining with Janus green.

7. Certain examples of "vital staining"—for instance, the staining of the axis cylinders of nerves with methylene-blue—are not purely vital stains, since the reaction can be elicited many hours after death.

2. TECHNIQUE OF VITAL STAINING

(i) **Methylene-Blue.**—The chief utility of this method is for the nervous system. A pure sample of this dye is essential. Its solubility in approximately 4 per cent. in water. The living animal may either be injected and the organs removed when sufficiently stained, or pieces of tissue may be treated in capsules with the dye.

Injection Method.—Of use especially when it is desired to stain tissues over a prolonged period, *e.g.* for experiments on phagocytosis. The solution, made up in isotonic saline or in Ringer-Locke solution, is injected intravenously or by the intraperitoneal route. The strength has to be worked out by the individual worker, since it depends both on the nature of the animal and on the nature of the observations. You may either inject a single (or several) massive doses at a high concentration (*i.e.* 1 to 4 per cent.) or give daily injections of a few c.c. at a smaller concentration (say 0·5 per cent.). The organs are then dissected out and treated as described below.

Immersion Method.—Thin slices of tissue are placed on glass wool in a capsule or deep Petri dish. The methylene-blue is added so as just to cover the tissues. There should be a large air-space above the tissues—*i.e.* between them and the cover of the vessel. Here again it is impossible to dogmatise on the right concentration of the dye, or the time required, to give a vital staining reaction.

A solution of 1 in 10,000 may first be tried ; if no staining occurs after thirty to sixty minutes it may be assumed that it is too weak, and a 1 in 1000 solution experimented with. It is better, when working with material of which you have no experience, simultaneously to stain fragments in solutions of 0·5 per cent., 1 in 1000, and 1 in 10,000. Sometimes good results are obtained with solutions as weak as 1 in 100,000.

Fragments of tissue are dissociated and examined with the microscope from time to time. Intravitam observations with methylene-blue must be made rapidly, since the dye becomes converted into its leuco-base soon after adding a cover-slip to the preparation. Should this occur, remove the cover-slip and expose the tissues to the air for a few moments, to reoxidise the leuco-base to methylene-blue.

For making permanent whole preparations, or sections, use the methods outlined on p. 277. But the fixation of methylene-blue (and other vital dyes) is not very satisfactory.

(ii) **Neutral Red.**—Really a basic dye, called neutral red because of its properties as an indicator : it turns yellow in the presence of alkalies. This is useful in intravitam observations on cell granules, etc. It is also one of the least toxic and most permeable of the vital dyes. Used especially for cell vacuoles and granules.

For work on mammalian tissues it is best added to the dissociated tissues on a slide. Strengths of 1 in 10,000 to 1 in 100,000 are usually employed. Another method, applied by Bensley to the pancreas, is to kill the animal by bleeding, and inject a 1 in 15,000 solution of the dye in isotonic saline. The method should be of use for other organs.

(iii) **Janus Green.**—This dye, as shown by E. V. Cowdry, may be almost regarded as a specific vital stain for mitochondria, provided that the concentration and the make of dye be right. The latter is most important : Janus green "*B*," made by Hoechst, should be used. Other varieties, or other makes (*e.g.* of Grübler) are said not to be so satisfactory.

Examine dissociated tissues in solutions of Janus green B in isotonic saline ; 1 in 10,000 is a useful concentration, but the specific mitochondrial stain may be given even by solutions as weak as 1 in 500,000. Janus green B stains mitochondria bluish green ; after some time they turn pink, and, finally, colourless. This is due to reduction of the dye to its leuco-base, which can be reoxidised by appropriate treatment.

In the case of the pancreas, Bensley obtained effective staining by injecting 1 in 10,000 Janus green through the aorta in animals previously bled to death. This technique could doubtless be applied to other organs.

(iv) **Isamine Blue** (=**Pyrrol Blue**).—A vital stain of low toxicity. Most tissues take it up after repeated injections of the dye into the animal. It may be injected by the subcutaneous or intraperitoneal routes in a 1 per cent. solution in isotonic saline. The dosage, according to Goldmann, is 0·5 to 1 c.c. per 20 grammes of body weight. Or it may be injected intravenously—an easy procedure in rabbits—in daily doses of 2 to 5 c.c. of a 0·5 per cent. solution. I have had very good results, with the organs deeply stained, after four or five injections each of 5 c.c. into the ear veins of rabbits. Another advantage of this dye is that it is well preserved by formol (preferably neutral). Frozen sections may be made, or even paraffin ones, though dehydration must be very rapid, as some of the dye is extracted. Saturated mercuric chloride in water can also be used as a fixative (von Moellendorf).

(v) **Trypan Blue.**—This gives similar results, and is used in the same manner.

PART IV

METHODS FOR SPECIAL ORGANS, TISSUES, AND CELL COMPONENTS

CHAPTER XIV

METHODS FOR SPECIAL ORGANS, TISSUES, AND CELL COMPONENTS

Adrenal Bodies—Alimentary Canal—Blood Films—Blood Vascular System—Bone and Decalcification—Cartilage—Centrosomes and Spindle Fibres—Chromaffin Tissue—Chromosomes—Connective Tissue—Ear—Embryos—Endothelia—Eye—Fat Globules—Golgi Bodies—Hair—Kidney—Ligament—Liver—Lung—Lymph Glands—Mammary Gland—Mitochondria—Muscle—Nail—Nervous System—Nucleoli—Ovary—Pancreas—Pigment Granules—Pituitary Body—Plasma Cells—Serous Membranes—Sex Glands—Skin—Spleen—Suprarenal Glands—Teeth—Tendon—Thymus—Thyroid—Uterus and Vagina—Yolk Granules and Discs.

ADRENAL BODIES

(See Suprarenal Glands, p. 293)

ALIMENTARY CANAL

For accurate histological work it is essential that the tissues should be fixed immediately after death—preferably while the body is still warm. The shrunken and fragmentary epithelial cells of the intestine and stomach, such as are encountered in most autopsy material, are due to post-mortem changes. Although such material is often serviceable from the standpoint of morbid histology, it is useless for cytological purposes.

Human Material.—If an autopsy is impossible immediately after death, putrefactive changes in the mucosa may be arrested by the injection of a large volume of the fixative down the œsophagus (in the case of the stomach) or by making

an incision in the abdominal wall and pouring in the fixing fluid (in the case of the intestines.)

Good fixatives for the mucosa of the alimentary tract are :

5 per cent. formol in 0·9 per cent. sodium chloride.
Mercuric chloride in saturated aqueous solution.
Mercuric chloride-formol.
Zenker's fluid.

Animal Material.—Here it is advantageous to distend the segment of intestine with the fixative. One end is ligatured ; at the other end, a loop is passed around a glass cannula which is connected to an all-glass syringe ; the fixative is injected so as moderately to distend the intestine. The end containing the cannula is then ligatured, and the whole segment is placed in a capsule of the fixative. In the case of the stomach the whole organ may be distended in this way. The alternative is to fix *segments* (NOT isolated pieces from the periphery, which curl) in the fixing fluid.

For cytological work, Flemming's fluid (with or without acetic acid) or Regaud's fixative are useful.

Embed in paraffin, bearing in mind the fact that the muscular coat is apt to become very brittle during clearing and embedding. Therefore clear in benzol or cedar oil (in preference to xylol), and reduce the paraffin bath to the minimum time consistent with permeation. The vacuum embedding method (see p. 55) is useful in this respect.

STAINING.—Standard histological methods are used. The oxyntic cells of the gastric mucosa are well shown by Mann's methyl-blue-eosin (see p. 134) in material fixed in mercuric chloride-formol ; also by Mallory's connective-tissue stain (p. 131).

FAT ABSORPTION can be studied in material fixed in Flemming's solution, counter-stained in safranin or eosin-methylene-blue (p. 133).

Mitrochondria are demonstrated by Heidenhain's iron-hæmatoxylin (p. 136) or the Kull method (p. 140), after fixation in Gatenby's modification of Flemming's solution (p. 39) or Regaud's fluid (p. 40). For the Golgi bodies of the epithelium, use the methods of Cajal or Da Fano.

BLOOD

Blood, owing to the fact that its constituent elements are in suspension in a fluid medium, the plasma, is studied almost entirely by the smear technique, the aim of which is to spread out a drop of blood in so thin a film that the cells or corpuscles are only one layer thick. The film is then fixed and stained. Most of the combinations of hæmatoxylin and the acid dyes can be applied to blood films, but such methods have now been well-nigh abandoned in favour of special blood stains, the aims of which are to stain in different colours the various elements of the blood. Not only are the normal constituents of the blood selectively stained by these methods, but so also are abnormal ones, such as myelocytes or parasites.

Romanowsky Method.—The methods used to-day are derivatives of the original Romanowsky method.

The basis of this is that if eosin and methylene-blue be dissolved together (usually with the aid of heat or an oxidising reagent) the resulting solution contains, in addition to the above two dyes, an oxidation product of the methylene-blue known as methylene-azure. The long known fact that an old methylene-blue solution stains better, and gives greater polychromasy, than a new one, is likewise due to the transformation of some of the methylene-blue into methylene-azure.

The very selective staining given by the Romanowsky stains is thus due to the presence of derivatives other than the original methylene-blue and eosin in the solution.

1. Fresh Preparations

Although the distinction between the different types of leucocyte is far easier in a stained film, the practised eye can identify them in a fresh preparation. In an emergency—*e.g.* in the absence of any stains—recourse can be had to this method, which is often a useful adjunct to the study of stained blood films.

To make a fresh preparation you proceed as follows :

1. Clean and dry an area of skin with ether or alcohol. With human beings the ball of the finger or the lobe of the ear are suitable areas.

In the case of the monkey, puncture the lobe of the ear ; for rabbits and cats, incise the edge of the ear with a fine scissors.

For rats and mice, the end of the tail is cut off with scissors and subsequently cauterised to stop the bleeding.[1]

In the case of hens, the axillary vein of the wing is punctured.

2. Obtain a drop of blood, preferably with a triangular blood needle. A small, sharp stab is requisite. In the case of thin-eared mammals, a small incision made with a fine scissors at the periphery is best. The instrument is sterilised by dipping the point in ether or high-grade alcohol. A quick squeeze is sometimes necessary in the case of human patients, especially if the circulation be faulty, to obtain the drop of blood. Do not massage or squeeze the finger (or lobe of ear) for long, as you will express lymph which will seriously dilute the blood. The drop of blood having been obtained, you proceed immediately with stage 3.

3. Take up the drop of blood by touching it with the centre of a perfectly clean slide. The drop should be small—about this size : ●

4. Immediately drop a square cover-slip ($\frac{7}{8} \times \frac{7}{8}$ inch) on the drop of blood. Flick, or gently tap, once or twice, the under-surface of the slide so as to flatten out the drop.

The slide is now ready for rapid observation, but, if you wish to examine for a long time, proceed to stage 5.

5. " Ring " the cover-slip with vaseline or one of the proprietary compounds sold for that purpose. In the case of vaseline, dip into a pot of this a ⊥-shaped metal rod, after heating in the flame. Apply quickly to the slide at the edges of the cover-glass. A hermetically sealed mount is thus obtained.

The " Deckglaskitt " made by E. Leitz is especially useful for sealing mounts of all sorts, and has the advantage over vaseline in that it sets very hard.

Notes.—(1) If the drop of blood be too large, the layer of cells between cover-slip and slide will be too thick for microscopical observation. The preparation should be colourless ; if pink, it is too thick.

(2) Do not touch the skin with the slide, only the periphery of the drop of blood. This is to avoid taking up epidermal squames or sweat.

(3) In the case of blood of mammals and birds, the study of move-

[1] A vivisection licence is necessary to do this in Great Britain.

ments of leucocytes, phagocytosis, etc., is only practicable with a warm stage.

(4) If you intend to employ the dark-ground illumination the slide must be of the requisite thickness (see p. 155).

2. Blood Films

Although staining a blood film is easy, the making of a good film is a matter of no little practice. A badly made blood film is like a badly fixed organ: no matter how brilliant the subsequent technique or (often) how painstaking the observation with the microscope, it will not yield the requisite information.

The following instructions cannot supply the "knack," but only the outlines of the procedure.

Provide yourself with a sufficient number of perfectly clean slides. The slightest trace of grease will render making a good film impossible. If the slides come from the maker in a fairly good condition, it will suffice to keep them in a glass-stoppered slide-pot of acid alcohol. If they are very dirty, you should soak them in the potassium bichromate-sulphuric acid cleansing mixture (formula, p. 120), then rinse in running and distilled water successively; and finally dip into 96 per cent. alcohol before drying with an absolutely clean duster.

1. Clean and disinfect the skin.

2. Prick and express a small drop of blood, as explained above.

3. Choose a slide with a well-ground and somewhat bevelled edge. Take up the drop of blood at one end of this.

4. Apply this slide, at an angle of 35° to 45°, at the surface of another slide, about 1 cm. (*i.e.* $\frac{1}{2}$ inch) from its end. Then push to the extremity. This requires a good deal of practice.

5. Dry the slide by waving it. This is especially important in a damp atmosphere. If you allow the water slowly to evaporate from the blood plasma, crenation of the red blood corpuscles will be caused by the gradual increase in concentration of the salts in the plasma.

The film is now ready for fixation and staining. Its naked-eye appearance should be translucent and even.

Notes.—When making blood films bear the following points in mind:

The pressure must be slight but even; the pushing movement fairly rapid—i.e. *the film should be spread out in about half a second.* Uneven movement of the "spreading" slide will produce uneven thickness of the film. The more the angle of the spreading slide approaches the vertical, the thinner the film, and *vice versa.*

If the slide shows areas over which no blood has been spread, this is due to grease on the surface. The slightest trace of fatty matter on the surface of a slide makes smearing impossible. With practice you will be able to gauge the size of the drop of blood so that the film will begin and end about 1 cm. (half an inch) from each end of the slide, although the pushing movement, though begun 1 cm. from one end, is continued to the extremity of the slide. It is important that the blood film should begin and end some distance from the ends of the slide, so as to facilitate observation of the extremities of the film.

Mounting Blood Films

Blood films are usually examined without a cover-slip with the oil-immersion objective. There are two main reasons for this: firstly, time is saved by omission of the mounting; secondly, the special blood stains fade fairly soon in balsam, owing to its going acid on keeping.[1] The oil is removed from the film after examination by washing the slide with xylol.

Staining Blood Films

1. Hæmatoxylin and Eosin

1. Fix in vapour of 40 per cent. formol for two to five minutes.
2. Plunge the slide into absolute alcohol for two to five minutes.
3. Bring down to water.
4. Stain in Delafield's or Ehrlich's hæmatoxylin. Usually stain for double the time required for sections—say ten to fifteen minutes.
5. Blue in tap water or tap-water substitute.
6. Stain in 5 per cent. eosin.

[1] But Romanowsky stains keep well in Gurr's neutral mounting medium. Blot and dry the film, then mount direct.

7. *Either* dehydrate, clear, and mount in dammar or balsam, *or* rinse in distilled water and blot. You can then examine the film direct (without mounting) with an oil-immersion objective.

Red blood corpuscles, red; nuclei or white cells, blue. Granules of eosinophils, pink; those of mast cells and neutrophils, unstained. Platelets, pink.

The results given by this technique are far inferior to those furnished by the special methods described below.

2. Leishman's Stain

Probably one of the simplest and most precise methods extant for staining blood for diagnostic purposes. For method of making up the stain, see p. 115.

Have ready a drop-bottle of the stain, a drop-bottle *and* a wash-bottle of distilled water, and some fluff-free blotting paper (or filter paper). Make a transverse line with a grease pencil across the slide at the beginning of the film.

1. Pour on n (say 8) drops of the stain on to the *unfixed* film. Leave one-half to one minute.

2. Add with the drop-bottle n to $2n$ (*i.e.* 8 to 16) drops of distilled water to the concentrated solution of the stain on the slide. Mix by gently inclining the slide first one way and then the other. Leave for ten to fifteen minutes.

3. Wash off the stain in a stream of distilled water from the wash-bottle. All precipitate should be removed in about fifteen seconds.

4. Blot the film.

5. *Either* mount the dry film in dammar or balsam, *or* examine direct with the oil immersion.

N.B.—Avoid at all costs bringing the stained film into contact with alcohol. If you do this, the stain will be extracted in a few seconds.

Red blood corpuscles, orange-pink. Nuclei of leucocytes, blue to purple. Granules: of eosinophils, pink; of polymorphs (neutrophils), purple; of mast cells (basophils), purple. Cytoplasm of lymphocytes, a slaty blue. Platelets, pink to purple.

The chromatin of malarial parasites, *Leishmania*, and

trypanosomes is stained red; the cytoplasm—in the case of the two former—blue. This reversal of the staining reaction, as compared with that of the leucocytes, aids the eye in the rapid detection of blood parasites. Bacteria are stained blue by Leishman's stain.

This stain is excellent for other cell-containing fluids.

NOTES.—(1) The reason why the undiluted stain is first poured over the blood film is to fix it—the stain being dissolved in absolute *methyl*-alcohol.

(2) The time required for staining after dilution of the stain with water, as also the number of drops of water added to the stain, has usually to be found out by the trial-and-error method for every new sample of the stain. Usually a dilution of the stain with an equal number of drops of water, and ten minutes staining by this is sufficient.

(3) It is essential to dissolve the stain in acetone-free methylic alcohol (see p. 115), also to dilute it with distilled water. If you use tap water the methylene-blue (and azure) components will not stain.

(4) Films which have been kept for months may be stained by Leishman's stain. The time of staining in such instances usually requires to be increased.

3. GIEMSA'S STAIN

Like Leishman, this is a mixture of methylene-blue, methylene-azure, and eosin. It stains more intensely than Leishman, but is slower.

This stain is best bought in solution from a reliable source (*e.g.* Gurr or Grübler).

1. Fix the film in absolute methyl or ethyl alcohol for two to five minutes.

2. Take two large watch-glasses; transfer the film—face downwards—to one of these. Add 5 to 10 c.c. of stain diluted in the proportion of 1 drop of stock solution to 1 c.c. of distilled water. The dilution must be done in a small graduated tube, *not* in the watch-glass. Then cover with the other watch-glass. Stain for twenty minutes to twenty-four hours. For staining of blood films for ordinary purposes twenty to thirty minutes will suffice; but the spirochætes—*e.g.* the *Treponema pallida*—require more energetic treatment (see p. 335).

3. Rinse in distilled water for several minutes.

4. Blot.

5. *Either* mount in dammar or balsam, *or* examine direct with the oil immersion.

Red blood corpuscles, bluish ; nuclei of leucocytes, purple. Eosinophil granules, red ; those of the polymorphs, reddish violet ; those of the mast cells, purple. The cytoplasm of lymphocytes is dark blue. Blood platelets, purple. Blood parasites and bacteria, as with Leishman.

4. Eosin-Methylene-Blue

There exist innumerable modifications of this method. Probably one of the best is that of Laveran. Borrel's methylene-blue, which enters into the composition of this stain, is easily prepared and keeps indefinitely. It is also obtainable from Gurr or Grübler. The mode of preparation is described on p. 110.

The following solutions are required :

(*a*) A solution of Borrel's methylene-blue diluted in the proportion of 1 of the stock solution to 9 of distilled water.

(*b*) A 1 in 10,000 solution of eosin in distilled water. The latter keeps badly, and is best prepared by the dilution of a more concentrated (1 per cent. or 5 per cent.) solution.

Titration of the Stain.—Take three watch-glasses, add to each of them one volume of solution A, then respectively add one, two, and three volumes of B. Mix and stain as described beneath. The slide in which the leucocytes appear a rich purple has been stained in the mixture most suitable for your purposes.

Staining.—1. Fix thin air-dried blood films in absolute alcohol for five to ten minutes.

2. Cover the films with the staining fluid without drying or washing them. Leave for ten minutes.

3. Rapidly rinse in water.

4. Blot.

5. *Either* examine direct with the oil-immersion objective *or* mount in dammar or balsam.

Red blood corpuscles, pink ; white blood corpuscles, in shades of blue and purple.

5. Wright's Blood Stain

This stain gives results very similar to Leishman's, and is widely used in the U.S.A. Like Leishman's stain it should be bought ready made up, as the preparation of stains of this type by the laboratory worker is a waste of time. As an alternative, buy the powder and make a 0·015 per cent. solution in pure methylic alcohol (*acetone-free*).

1. Make thin air-dried blood films.
2. Flood the unfixed film with the undiluted stain. Leave for two to three minutes.
3. Wash in running water for about thirty seconds. The film should be pale pink.
4. Blot.
5. *Either* examine direct with the oil-immersion lens *or* mount in dammar or balsam.

Staining is very similar to Leishman's stain.

Cell-containing fluids other than blood may be stained by this method.

BLOOD VASCULAR SYSTEM

Small vessels should be fixed by stretching on a piece of filter paper, which is then placed face downwards in a dish of fixative. They will adhere to the paper during fixation. Larger vessels are cut into segments which are thrown into the fixing fluid. The ends roll up, and hence should be trimmed before embedding. Inclusion in paraffin gives good results, provided that prolonged treatment with high-grade alcohol be avoided.

The endothelium peels off soon after death. It is fragmentary in most of the material taken from the post-mortem room. Extreme contraction of the vessel, on being placed in the fixative, may produce a similar result.

The same remarks apply to sections of the heart. The tendency of large pieces of cardiac muscle to become brittle may be avoided by clearing in benzol or cedar oil. Very large pieces of heart are best embedded in celloidin.

Suitable Fixatives

Mercuric chloride-formol.
5 per cent. formol in 0·9 per cent. sodium chloride.
Bouin's fluid.
Zenker's fluid.

Suitable Stains

Hæmatoxylin and eosin.
Heidenhain's iron-hæmatoxylin.
Hæmatoxylin and van Gieson (to differentiate between collagen and muscle fibres).
Weigert's elastin stain (to show elastic tissue).
See also Serous Membranes and Endothelia, p. 282.

BONE AND DECALCIFICATION

The calcium salts of bone form an insuperable bar to section-cutting. Although ground bone sections are used for demonstration purposes, the cells of bone are destroyed by the grinding process, and the method is not suitable for histological observations. For such work the calcium salts are dissolved out by treatment with an acid, leaving behind the organic matrix of bone—ostein—(which can easily be sectioned), and the cellular elements of bone.

The essential steps in decalcification involve :

(i) Thorough fixation of the tissues in a non-acid fixative.
(ii) Decalcification in an appropriate acid solution.
(iii) Removal of the acid—" De-acidification "—by treatment with an alkaline solution, but not by prolonged treatment in tap water, since this is apt to produce swelling of collagen fibres if used immediately after the acid.
(iv) Washing in water to eliminate the alkali.

The criteria of a good decalcifying solution are :

(i) Complete removal of the calcium salts.
(ii) The absence of harmful effects on cells and connective-tissue fibres.
(iii) The absence of harmful effects on staining processes.

1. Fixation

Any non-acid fluid may be used; 5 per cent. formol in 0·9 per cent. sodium chloride and the mercuric chloride-formol mixture advocated in this book are excellent.

Fix thoroughly thin slices of bone, except in the case of fœtal bones which can be fixed *in toto*. A fine saw should be used for excising slices of adult bone which should (preferably) not be more than a few millimetres thick.

2. Decalcification

The following fluids are good:

Formol-Nitric Acid

5 per cent. formol aqueous solution . .	100 c.c.
Nitric acid (1·4 specific gravity) . .	7·5 to 15 c.c.

1. Place the tissues in a large volume of this fluid and change once or twice a day. When bone can be easily punctured with a needle, and has become flexible, decalcification is complete. In the case of young animals this takes, in my experience, twenty-four to forty-eight hours. The formol inhibits any tendency towards maceration on the part of the nitric acid.

2. Remove the acid by treatment with a 5 per cent. aqueous solution of sodium sulphate (Na_2SO_4), lithium sulphate (Li_2SO_4) or potash alum [$K_2SO_4.Al_2(SO_4)_3.24H_2O$] for twelve to twenty-four hours.

3. Then wash twelve to twenty-four hours in running water.

Phloroglucin-Nitric Acid

This method is to be recommended for rapid decalcification; for fine histological work the slower formol-nitric acid method is preferable.

Place the tissues in a large volume of the decalcifying fluid which is prepared as follows:

Add slowly to 1 gramme of phloroglucin, in a porcelain capsule, 10 c.c. of concentrated nitric acid. When the ebullition has ceased, add 100 c.c. of 10 per cent. nitric acid.

Decalcification is complete in a few hours. The fluid, at

first dark red, turns yellow a few days after being prepared. But it is equally efficacious. The phloroglucin protects—in a way not understood—the tissues from the swelling and macerating action of the acid.

Wash well in running water (twelve to twenty-four hours).

Sulphurous Acid (H_2SO_3)

Decalcify in a saturated (5 per cent.) solution of this. Bones of small mammals are decalcified in twenty-four hours; for large human bones treatment should be prolonged for one week. This method has also been recommended for decalcifying teeth.

Wash for twenty-four to forty-eight hours in running water. The best results are said to be obtained after formol fixation.

Flemming's Fluid

Only to be recommended for the decalcification of very small pieces of tissue. The solution must be frequently renewed. Then wash in running water for twelve to twenty-four hours.

Many other decalcifying fluids have been recommended, but the above methods suffice for all requirements.

Note.—*For very delicate objects*, such as the cochlea, the following method is useful:

(1) Fix in Flemming's or Regaud's fluid.
(2) Wash in running water for twelve hours.
(3) Dehydrate and embed in celloidin.
(4) Place the trimmed celloidin block in water until it sinks.
(5) Delcacify in dilute (3 to 5 per cent.) nitric acid.
(6) Treat with 5 per cent. lithium or sodium sulphate for twenty-four hours.
(7) Wash in running water for twenty-four hours.
(8) Dehydrate in ascending grades of alcohol to 85 per cent.

The advantage of this method lies in the protection afforded during decalcification to delicate tissues by the celloidin.

3. Embedding

For rapid work with small pieces of bone paraffin embedding is quite satisfactory. For large specimens, celloidin enables better sections to be cut.

4. Staining Methods

(*a*) General Stains

Hæmatoxylin (of Ehrlich or of Delafield) and Mallory's connective-tissue stain give good pictures of ossification. With Heidenhain's iron-hæmatoxylin the ossein matrix is stained black. Nicolle's carbol-thionin gives excellent pictures of ossification, with the ground substance of cartilage metachromatically stained purple.

(*b*) Special Stains

Schmorl's Picro-Thionin Method.—The method depends on the precipitation of the stain in the bone canaliculi. Picric acid combines with many aniline dyes (including thionin) to form precipitated picrates. It is excellent for demonstrating bone canaliculi.

Fix in formol or Regaud's fluid. Avoid fixatives containing mercuric chloride, as the stain does not succeed after these. Decalcify in the formol-nitric acid mixture (p. 210). Embed in celloidin or make frozen sections.

Stain sections as follows :

1. Wash in water for ten minutes.
2. Stain in Nicolle's carbol-thionin for five to ten minutes.
3. Rinse in water.
4. Treat with a saturated aqueous solution of picric acid for one-half to one minute.
5. Rinse in water.
6. Differentiate in 70 per cent. alcohol until the stain ceases to pour out from the sections—usually five to ten minutes.
7. Dehydrate in 96 per cent. alcohol.
8. Clear in origanum oil or carbol-xylol (formula, p. 147).
9. Mount in dammar or balsam.

Ground substance, yellow to brown ; bone canaliculi, dark brown to black ; cells, red ; ground substance of cartilage, a brilliant purple.

Note.—Should the stain fail, make the thionin alkaline by adding 1 to 2 drops of ammonia to it. This is best done to a few c.c. of stain

to a test-tube at the moment of using it, as the alkaline solution does not keep.

Schmorl's Thionin-Phosphotungstic Acid Method.—Another very useful method for demonstrating the general structure of young bones and ossification. It is said only to be suitable for the bones of children, but I have had excellent results with the bones of young mammals.

Fix in formol. Decalcify in the nitric acid-formol mixture (p. 210).

Dehydrate and embed in celloidin, and cut sections as thin as possible. Stain as follows :

1. Wash the sections in water for ten minutes.
2. Stain in a half-concentrated aqueous solution of thionin for ten to thirty minutes.
3. Rinse in water.
4. Differentiate the stain in 70 per cent. alcohol.
5. Rinse in water.
6. Treat with a concentrated aqueous solution of phosphotungstic or phosphomolybdic acid for a few seconds.
7. Wash the sections in water until they are a bright transparent blue. This usually requires five to ten minutes.
8. Fix the stain in 5 or 10 per cent. formol for one to two hours.
9. Wash in running water for three to twelve hours.
10. Dehydrate in 70 per cent. and 90 per cent. alcohol.
11. Clear in carbol-xylol or in the acetone-xylol mixture (formulæ, p. 147).
12. Mount in dammar or balsam.

Ground substance (ossein), pale blue or unstained ; cells and nuceli, blue ; canaliculi, bluish black ; ground substance of hyaline cartilage, purple.

CARTILAGE

Fix in mercuric chloride-formol or 5 per cent. formol. In the case of senile (calcified) cartilage decalcification (*q.v.*) may be necessary. Embed in paraffin or celloidin.

Suitable stains, in addition to hæmatoxylin (of Delafield or of Ehrlich) and eosin are : Nicolle's carbol-thionin ; Borrel's

methylene-blue ; safranin. All of these give a metachromatic staining of the ground substance of hyaline cartilage ; the two former stain it purple, the latter yellow. For methods for showing the changes in cartilage during ossification, see the section on Bone.

The elastic fibres of elastic cartilage are demonstrated by Weigert's elastin and the other specific stains for this type of fibre.

The collagen fibres of white fibro-cartilage are stained by van Gieson or the Ponceau S substitute.

CENTROSOMES AND SPINDLE FIBRES

Fixation in mercuric chloride (with or without acetic acid), Bouin's fluid, or Flemming's fluid is suitable.

Generally speaking, those fixatives which preserve the cytoplasmic inclusions (*e.g.* the fluids of Regaud, Champy, and Gatenby's Flemming without acetic acid) should not be used for demonstrating centrosomes, since the preservation and subsequent staining of the various cytoplasmic constituents merely hinders observation.

As recommended by Bolles Lee, quite the best stain is Heidenhain's iron-hæmatoxylin. Differentiation must be carefully controlled with the staining microscope. A counter-stain should not be used, as it usually masks the clear-cut images furnished by the iron-hæmatoxylin. Thin (*i.e.* not more than 5 μ) paraffin sections are requisite.

CHROMAFFIN TISSUE

(See Suprarenal Glands, p. 293)

CHROMOSOMES

For all work on counting chromosomes, tissues should be perfectly fresh. Thin slices of tissue, made with a very sharp knife, or a safety-razor blade held in an artery forceps, are required for fixation. Crushing or treatment of tissues with saline or other solutions must be avoided.

The following fixatives are suitable for counting chromosomes :

Carnoy's fluid.

Sansom's modification of this (p. 296).

Bouin's fluid.

Flemming's fluid with acetic acid (for very thin fragments of tissue).

Many other fluids, some of which contain urea, have been recommended, but their superiority is doubtful.

The duration of the fixing bath must be ascertained by experiment. Often better results may be obtained by fixing either in the ice-chest or by warming the fixative to body temperature. For research purposes several methods of fixation should be tried until a suitable technique has been evolved.

After-Treatment.—Careful dehydration, clearing, and embedding in paraffin should be followed by *serial sections of a suitable thickness.* The latter is governed by the size of the cells and chromosomes, the larger the cells the thicker the sections. For Mammalian chromosomes, sections of 5 to 10 μ are usually indicated.

Staining.—Most work on chromosomes is done with Heidenhain's iron-hæmatoxylin. Careful differentiation, until the chromosomes are black or blue-black, and the surrounding cell substance practically colourless, is necessary. A water immersion objective is helpful here. Counter-staining is usually more of a hindrance than a help.

Delafield's hæmatoxylin, followed by differentiation in dilute hydrochloric acid, is used by some. So also are thionin, toluidin blue, and gentian violet. These dyes are best differentiated in 90 per cent. alcohol.

A good plan, when examining material in which the presence of mitosis is not certain, is to stain by Monica Taylor's method in thionin (toluidin blue or gentian violet are equally good), mount in dammar or balsam, and examine the material immediately. If the material warrants further examination, dissolve the mount in xylol and restain in iron-hæmatoxylin. I prefer to examine after the thionin in cedar oil ; in the latter case the sections need not be covered if the oil immersion be used.

Counting of chromosomes is aided by plotting them out with an Abbe camera lucida. Continuous and careful focusing is needed. The section before, and the section after, the one containing the cell under study should also be examined, so as to make certain that all the chromosomes are included.

CONNECTIVE TISSUE

Both collagen and elastic fibres continue to give their specific staining reactions for many hours after death.

A. Collagen Connective Tissue

No special fixative need be used, though Mallory's fuchsin-aniline blue-orange G stain gives the best results after Zenker's fluid. For other staining methods, 4 per cent. formol in 0·9 per cent. sodium chloride, mercuric chloride-formol, and Bouin's fluid give good results.

The general staining reaction of collagen fibres is oxyphil.

Suitable Stains

Van Gieson's Picro-Fuchsin Stain. (See p. 129.)

The Ponceau S substitute for van Gieson (p. 129), though less reliable for the identification of young collagen fibres, has the advantage of not fading.

Schmorl's van Gieson-Carbol-Xylol Technique.—I have found this technique better than any other for clearly identifying collagen fibres. It is particularly useful for revealing an incipient fibrosis.

1. Stain in Heidenhain's iron-hæmatoxylin.
2. Stain in van Gieson's picro-fuchsin, modified as follows :

Saturated aqueous solution of picric acid	100 c.c.
1 per cent. aqueous solution of acid fuchsin	10 c.c.

3. Rinse rapidly in distilled water.
4. Transfer direct to 96 per cent. alcohol for about thirty seconds.
5. Clear in carbol-xylol (formula, p. 147)—a few minutes will suffice.

6. Rinse rapidly in pure xylol.
7. Mount in dammar or balsam.

Nuclei, black or greyish; collagen fibres, bright red; epithelia and muscle, yellow; red blood corpuscles, black; neuroglia, yellowish.

NOTE.—Owing to the acidity of carbol-xylol ordinary hæmatoxylin mixtures (*e.g.* of Ehrlich) are destained. Nuclear staining with the basic aniline dyes is impossible for the same reason.

Mallory's Fuchsin-Aniline Blue-Orange G Method (p. 131) is very useful, but less specific than the above. Collagen fibres, blue.

Unna's Safranin-Water Blue-Tannin Technique.—Fix (preferably) in alcohol, or in a saturated aqueous solution of picric acid, and make celloidin sections. Formol or Bouin fixation must not be employed.

NOTE.—The method can be applied to paraffin sections.

1. Stain for ten minutes in a 1 per cent. aqueous solution of safranin.
2. Wash thoroughly in water.
3. Stain for ten to fifteen minutes in the following, *freshly mixed*, solution:

1 per cent. aqueous solution water blue .	Equal parts.
33 per cent. aqueous solution tannin .	

4. Wash thoroughly in distilled water.
5. 96 per cent. alcohol.
6. Bergamot oil (or absolute alcohol, then xylol, for paraffin material).
7. Dammar or balsam.

Nuclei, red; collagen fibres, blue.

B. Elastic Fibres

Weigert's Elastin Stain (p. 130) is excellent for general use, and is profitably combined with safranin or neutral red as nuclear stains. Elastic fibres, dark blue to black. The resorcin-fuchsin solution deteriorates with age; the phenol-fuchsin variant keeps longer, and is better.

Unna-Taenzer Method.—Another very precise stain for elastin.

Fix in alcohol, formol, mercuric chloride-formol, or Müller-formol (=Regaud's fluid).

Make frozen, paraffin, or celloidin sections.

1. Stain for two hours at 37° C., or for twelve to twenty-four hours at room temperature, in the following solution :

Orcein *D*	1 grm.
Concentrated hydrochloric acid .	1 c.c.
Absolute alcohol	100 c.c.

2. Wash in alcohol till no more stain pours out from the sections.

3. Destain in hydrochloric acid alcohol (0·5 per cent. hydrochloric acid in 70 per cent. alcohol).

4. Dehydrate, clear, and mount in dammar or balsam.

Elastic fibres, dark red to dark brown.

E. Fraenkel's Method.—See p. 291. This technique is particularly good for the dermis.

Van Gieson and Weigert's Stains.—A useful method for demonstrating both collagen and elastic fibres in the same section. See p. 131.

EAR

External Ear (Pinna).—Use standard methods. The elastic fibres are well shown by Weigert's elastin stain and the other specific stains for elastin.

Internal Ear. — The cochlea demands special treatment ; the organ of Corti in particular demands excellent technique if shrinkage of its cells and rupture of the membrana tectoria is to be avoided. Fixation soon after death is essential.

The cochlea of the guinea-pig is particularly easy to excise owing to the thinness of the surrounding bones, and the following method, advocated by Schäfer, is also applicable to other subjects than the guinea-pig.

Prolong the mouth aperture by cutting through the muscles on each side with strong scissors. Then tear away the lower jaw so as to expose the base of the skull. The tympanic bulla will be seen just behind the articulation of the jaw. Cut through the external auditory meatus,

and remove the bulla from the skull by breaking through the petrous bone. Make an aperture in the bulla and locate the cochlea, which appears as a thin-walled conical projection. Then cut down the bulla with strong scissors till the cochlea can be isolated from it. Remove the cochlea and carefully free its attached end from the bone of the bulla.

Then fix it by one of the following methods recommended by van der Stricht:

1. 5 *per cent. Aqueous Solution of Trichloracetic Acid.*—Fix for one to three days. This fluid both decalcifies and fixes.

Transfer the cochlea direct to absolute alcohol to which a little iodine has been added. Embed in paraffin.

2. *Zenker's Fluid.*—Fix for one to two days. Wash in running water for twelve hours. Then harden in 70 per cent. alcohol to which a little iodine has been added. If necessary, decalcify in 2 per cent. nitric acid in 70 per cent. alcohol. Embed in paraffin.

3. *Bouin's Fluid.*—Fix for one or two days. Treat as for 2, but omit the iodine.

To obtain a good cytological fixation of the membrana tectoria it is necessary, after making one or two openings in the wall of the cochlea, *either* to fix the organ for fifteen minutes in the vapour of 1 per cent. osmic acid, *or* to immerse it for one hour in the same. Then proceed with post-fixation by one of the above methods. Mitochondria are well preserved by this technique.

Note.—The treatment with iodine-alcohol not only removes the precipitate of mercuric chloride (in the case of Zenker's fluid), but acts, according to van der Stricht, as a mordant for the following staining method:

Stain paraffin sections on the slide thus:

1. 2·5 per cent. iron-alum solution, one day.
2. Distilled water, one second.
3. Treat for a few minutes with an 0·5 per cent. solution of Congo red.
4. Rinse the sections in distilled water.
5. Stain in 0·5 per cent. hæmatoxylin, one day.
6. Differentiate in the iron-alum solution.

7. Wash for one hour in running water.

8. Dip the sections, for a few seconds only, in an 0·25 per cent. solution of lichtgrün in 95 or 96 per cent. alcohol.

9. Dehydrate in absolute alcohol.

10. Clear in xylol.

11. Mount in balsam.

Nuclei, central corpuscles, and terminal bars, blue-black to black ; cytoplasm, red ; connective tissue and membrana tectoria, green.

NOTES.—(1) I find that stages 1, 5, and 7—especially when the first period of fixation in osmic acid is avoided—may be greatly curtailed. One hour's mordanting in iron alum, an equal time in the hæmatoxylin, and ten minutes' washing in running water are more than sufficient.

(2) No matter what counter-stains (if any) are adopted, it will generally be found that Heidenhain's iron-hæmatoxylin, followed by careful differentiation with the staining microscope, gives the best preparations of the organ of Corti.

(3) For adult cochleæ, where decalcification is essential, the method of decalcification *after inclusion in celloidin* is sometimes useful. See p. 211 for an account of this.

EMBRYOS AND OVA

Material should be fixed as soon after death as possible. The human embryologist, however, has to be content with what he can get, and good work has been done with aborted embryos which have only been fixed many hours afterwards.

Ovary and Uterus.—See the special sections on pp. 284 and 299 respectively.

Ova: Early Stages.—These are encountered in the Fallopian tube. There are two ways of obtaining them for microscopical study : one is to open up, or wash out, the tubes and to remove the isolated ova. The other is to fix the tubes entire, and then examine serial sections of them.

Isolation Method.—Either wash out the tubes from the fimbriated end with warm saline or slit them up in the same medium. Examine for ova, on a black background, with a binocular dissecting microscope. This method is only applicable to medium-sized and large mammals.

Sectional Method.—Fix (*vide infra*) the tubes entire; cut them into segments, and make serial sections—a tedious process. Sometimes this method is necessary for very small mammals (*e.g.* mice).

Fixation of Ova.—Most of the standard fixatives have been recommended by various authors. Owing to the smallness and delicacy of these objects, formalin, when used, should be well diluted—2 to 3 per cent. in isotonic saline.

Other fixatives are: Zenker's fluid, and Regaud's potassium bichromate-formol.

For the ova and segmentation stages of marsupials, J. P. Hill's fluid should be used.

Distilled water . .	100 c.c.	= Solution A.
Nitric acid . .	5 c.c.	(Mayer's picro-nitric
Picric acid crystals to saturation		fixative.)
1 per cent. osmic acid . .		= Solution B.

To prepare Hill's fluid mix 96 c.c. of A with 2 c.c. of B.

To this, 2 c.c. of glacial acetic acid may be added. For delicate work it is best left out.

For cytological observations on ova and segmentation stages, standard cytological methods should be applied.

Embryos.—Most fixing fluids give good results.

Three to 4 per cent. formol penetrates well and has the advantage that embryos may be preserved in it for many weeks without harm. Mercuric chloride-formol and Zenker's fluid are also good.

Embedding of Embryological Material.—Careful dehydration in upgraded alcohols is necessary in order to reduce shrinkage as much as possible. Even more important is the clearing process in the case of paraffin inclusion. Reagents which tend to harden or make tissues brittle—such as xylol—should be avoided. The direct transference from the clearing medium to pure molten wax must *never* be made with embryological material. Always pass such tissues through a mixture of clearing medium plus paraffin. J. P. Hill's method is thoroughly recommendable.

1. Place the dehydrated material overnight in oil of cedar. This gives a gradual substitution of the oil for the alcohol.

2. Transfer to benzene for one or more hours.

3. Place in a tube of benzene to which paraffin shavings have been added. Leave the tube overnight (uncovered) on the top of the thermostat.

4. Embed in paraffin in the usual manner.

The Inclusion Mass.—The deformation of tissue layers during the "flattening out" process of paraffin sections, together with the greater shrinkage produced by paraffin than by celloidin, are points against the paraffin method.

Certain embryological stages are very rarely seen, particularly in human material, and hence the surest method of obtaining satisfactory sections — the celloidin technique — should be used.

Double Embedding is particularly indicated for embryological work owing to the ease with which serial sections are obtained. The technique is described on p. 62.

Such double-embedded sections require careful treatment to secure adhesion to the slide. Hill and Wilson recommend the following method :

1. Flatten out in the ordinary glycerine and albumen solution.

2. Dry the slides thoroughly.

3. Dip the slides into an 0·5 to 0·75 per cent. solution of celloidin.

4. As soon as the film has dried, place them in a slide jar containing :

90 per cent. alcohol . . .	9 parts.
Chloroform	1 part.

N.B.—Wherever alcohol of 90 per cent. or more is used (*e.g.* during dehydration), it should contain 10 per cent. of chloroform to prevent solution of the celloidin film. Staining is very slightly slowed down.

Orientation of embryos is most important. When these are not too small it may be done in the ordinary way, but for small specimens some mode of securing the orientated embryo to a piece of soft tissue must be employed. Hill's technique is a useful way of orientating embryological material. Here the tissues are stuck on to a square of pig's fœtal membrane with

a little dilute photoxylin (or celloidin) solution. The whole is then placed in chloroform-alcohol (*vide supra*) to harden the celloidin. Liver or brain may be used instead of the fœtal membrane.

ENDOTHELIA

(See Serous Membranes, p. 282)

EYE

The fact that the eye is a composite organ, containing many tissue layers which tend to separate during fixation and sectioning, and also that large sections involving the entire globus oculi are often requisite, renders it necessary to take certain precautions when preparing specimens for microscopic examination.

The sooner the organ is fixed after death (or operation) the better, as post-mortem changes appear fairly soon in the retina, while the cornea drys and diffusion of the intra-ocular fluid sets in.

The following methods are suitable for making sections through the whole organ:

Formol Fixation

1. Fix the entire eye in 4 per cent. formol in 0·9 per cent. sodium chloride. Do not prolong fixation beyond twenty-four hours, or the sclera and lens will become extremely tough. Mr. William Chesterman, of the Anatomical Department, Oxford, who has had considerable experience in the matter, tells me that Schering's formalin gives results undoubtedly superior to those of other makes. The latter often cause the retina to separate from the choroid. Furthermore, stage 3 (the freezing of the eye) is rendered difficult.

Another solution recommended by Greef is:

Schering's formalin	100 c.c.
Sodium chloride	6 grms.
Distilled water	1000 c.c.

2. Rinse the eye in running water.

3. To open the eye proceed as follows:

Wrap the eye in a very thin gutta-percha membrane.

Place this in a freezing mixture of crushed ice (2 parts) and common salt (1 part) for at least half an hour. Then divide the eyeball with a very sharp knife in the desired plane (usually antero-posterior).

4. Place the two halves in distilled water to thaw them.

5. Dehydrate in ascending grades of alcohol, beginning with 50 per cent. alcohol, and increasing each grade by 5 per cent.

6. Embed in celloidin by the " wet " process (see p. 61).

7. Cut sections. A suitable thickness is 15 to 25 μ.

8. Stain sections in hæmatoxylin (of Delafield or of Ehrlich) and eosin; or in Mann's methyl-blue-eosin, or in Mallory's connective-tissue stain. Celloidin sections of eye should be cleared by the creosote method (p. 146).

The above method produces the minimum of distortion of the globe.

For the retina, the following method is good :

1. Fixation in Zenker's fluid (formula p. 36) for twelve to twenty-four hours.

2. Wash out the uncombined potassium bichromate with running water for twelve hours. Then proceed with stages 3 onwards as above.

Note.—Mr. Chesterman tells me that while Zenker's fluid gives a good general fixation, the ciliary body is nearly always stripped away during sectioning, presumably because of undue hardening of its muscle. But excellent pictures of the cell layers of the retina are obtained by staining thin paraffin sections in Heidenhain's iron-hæmatoxylin.

Fixation in Flemming's Fluid (formula, p. 38).—Fix the eye for one to three days in this. Then rinse overnight in running water. Embed in paraffin and stain either with Heidenhain's iron-hæmatoxylin or with the aniline dyes. This method of fixation is often used for the retina ; it is *not* suitable for sections through large masses of tissue.

Embedding.—For large sections, involving the whole (or the major part) of the eye, celloidin is to be preferred. This is because it enables flat and unbroken sections to be cut. For thin sections of small segments of the eye, or of retina only, paraffin is more suitable, since it is very difficult to cut celloidin sections thinner than 15 μ.

When embedding in paraffin, the use of ligroin (petroleum ether) is strongly recommended by Langeron, since this re-

agent hardens tissues far less than xylol, toluol, and the other common clearing media. The material should always be passed through a mixture of absolute alcohol plus the clearing medium; then into the clearing medium and finally into a mixture of the clearing reagent plus paraffin. Treatment with successive baths of pure paraffin can then be begun. Violent ex- or end-osmosis must be carefully avoided in the treatment of the eye.

Other special methods applicable to the eye are:

The Weigert methods for medullary sheaths (of use in degenerative processes of the optic nerve). See p. 255 *et seq.*

Methylene-blue (for the study of the ganglion cells of the retina). See pp. 242 and 243.

Golgi's impregnation methods (for neuroglia, ganglion cells, and their processes). See p. 251 *et seq.*

The neuroglial methods (for the neuroglia fibres of the optic nerve and retina). See p. 264 *et seq.*

For the study of the lens Greef advises either weak (2 per cent.) formol or Zenker's fluid. Celloidin embedding is essential owing to the hardness of this structure.

For further technical refinements in the study of the eye, consult the admirable handbook by Greef, Stock, and Wintersteiner (*Guide to the Microscopic Examination of the Eye.* English translation by Walker, 1913).

For the lens, fixation in the following solution is recommended by Szent-Gyorgi:

Acetone	125 c.c.
Glacial acetic acid	5 c.c.
40 per cent. formalin	40 c.c.
Distilled water	100 c.c.
Mercuric chloride crystals	4 grms.

Small lenses are immersed for two to three days in this fluid; larger ones require six to seven days fixation. Then add 50 c.c. of acetone for every 100 c.c. of fixative, and treat the tissues for a further two to three days. Embed in celloidin.

FAT GLOBULES

See "Histo-Chemical Methods," section on fat and lipoids, p. 167.

GOLGI BODIES

(= Golgi Apparatus ; Golgi Reticulum, etc.)

Suitable methods :
Cajal's uranium nitrate-formol method, p. 142.
Da Fano's modification of this, p. 143.
Golgi's arsenious acid method, p. 243.

For an excellent review of other methods, see Da Fano ("Methods for the Demonstration of Golgi's Internal Apparatus." *Med. Science Abstracts*, 1925, vol. xi. p. 319).

HAIR

(See SKIN, p. 292)

KIDNEY

For cytological work on the renal epithelium the organ should be quite fresh ; for other purposes, kidney is fairly resistant to post-mortem changes.

Mercuric chloride-formol, 5 per cent. formol in 0·9 per per cent. sodium chloride, and Zenker's fluid give a good fixation. Bouin's fluid must always be avoided, for it fixes this organ execrably.

All general staining methods are applicable. For the application of the special stains for fat and lipoids see "Histo-Chemical Methods," p. 167. The mitochondria are well shown by the Regaud-Kull technique, the Golgi elements by the Cajal method or Da Fano's adaptation of it.

LIGAMENT

(See TENDONS, p. 297)

LIVER

Post-mortem changes, detrimental to cytological observations, occur very soon after death. When taking pieces of

liver for micro-anatomical study from the cadaver, those areas of the organ which are in contact with the alimentary tract should be avoided, as it is here that post-mortem changes first appear. The greenish discoloration, often seen in such areas in both human and animal livers, is due to the liberation of hydrogen sulphide from the intestine, and the consequent breaking down of hæmoglobin to form iron sulphide. Furthermore, bacteria, and especially *Bacillus coli*, are often found in the liver at autopsy, as a post-mortem invasion of the organ.

Suitable fixatives for general work are :

4 per cent. formol in 0·9 sodium chloride.

Mercuric chloride-formol.

Mercuric chloride in saturated aqueous solution.

Zenker's fluid.

Both formol and mercuric chloride preserve bile.

The mitochondria are well shown by Heidenhain's iron-hæmatoxylin following formol fixation. The Kull method and Baker's modification of it (see p. 140) are also excellent.

The demonstration of the following substances is dealt with in the chapter on histo-chemical methods :

Fat, p. 167.

Glycogen, p. 173.

Pigments, p. 178.

Bile Canaliculi.—A simple way to show these is to fix in 4 per cent. formol in 0·9 per cent. sodium chloride, make paraffin sections of 8 to 10 μ, and overstain somewhat with Heidenhain's iron-hæmatoxylin. The canaliculi appear under an oil-immersion objective as fine, unstained tubules and clefts between the darkly stained hepatic cells.

Another technique, which has the advantage of giving a positive impregnation of the canaliculi, is to apply the Golgi potassium bichromate-silver nitrate method.

Schäfer advises the following modification :

1. Fix small fragments of liver for several weeks in 2 per cent. potassium bichromate.

2. Transfer direct to a 1 per cent. solution of silver nitrate—which should be changed at least once—for twelve to twenty-four hours.

3. Dehydrate, clear, and embed in paraffin.

4. Mount the sections in balsam.

A black deposit is formed around and in the canaliculi; the outermost regions of the sections are usually overimpregnated.

LUNG

The lungs are fairly resistant to post-mortem changes; when these occur, the first indication, in sections, is the desquamation and shrinkage of the bronchial epithelium.

Lung substance requires special treatment, because the air which it contains has to be removed before it can be satisfactorily embedded. Further, if you wish to have any idea of the inter-relationships of the air chambers and of their size, the pulmonary collapse caused by opening the thoracic cavity must be diminished. The following methods are good. The smaller the animal the better. Kill by a blow on the head, or by chloroform anæsthesia followed by section of a carotid.[1] Animals which are killed under anæsthesia show intense pulmonary congestion, and, in the case of small rodents in which the lungs are particularly delicate, actual hæmorrhage into the alveoli may be caused. This may be avoided by opening a large blood vessel; with rabbits and guinea-pigs the operation must be rapidly performed, or the animal dies under chloroform before the vessel is severed.

1. Injection Fixation. — *Without* opening the thoracic cavity, expose the trachea, insert a cannula, and inject the fixative either with an all-glass syringe or by gravity. Stop the injection as soon as moderate resistance is encountered. Tie off the trachea. The lungs, heart, and trachea are then dissected out *in toto* from the thorax and placed in a jar of fixative.

2. Immersion Fixation. — Ligature the trachea before opening the thorax. Remove the organs as above. Then place them in the fixative with a pad of cotton wool above the lungs to prevent their floating.

3. Isolated Pieces of Lung, such as obtained from the post-mortem room, are thrown into the fixative.

[1] A vivisection licence is necessary to do this in Great Britain.

Suitable fixatives are :

Mercuric chloride-formol.

5 per cent. formol in 0·9 per cent. sodium chloride.

Bouin's fluid.

Zenker's fluid.

The first of these has given me the best results, though ordinary formol is very good. Both these fixatives are useful in that red and white blood corpuscles are well preserved. Prolonged fixation—twelve to forty-eight hours—is needed. With methods 1 and 2 the lungs are cut into fragments suitable for sectioning when in alcohol of 50 or 70 per cent.

Removal of the air from the pieces of lung may be effected by keeping them in 70 per cent. alcohol until they sink ; if you do this in the incubator at 37° C. the process is somewhat accelerated, but even so it usually takes days. The use of the vacuum embedding bath is strongly recommended, but care must be taken to avoid causing artefacts by a too rapid exhaustion (see p. 55 for a description of the method). Pneumonic or otherwise consolidated lungs can be embedded in the ordinary manner.

Standard staining methods can be used. Incipient fibrosis is best shown (in my experience) by Heidenhain's iron-hæmatoxylin and van Gieson, the latter used according to Schmorl's method (see p. 216). The elastic fibres can be satisfactorily shown by safranin and Weigert's elastin stain.

For fat emboli, see p. 312.

Alveolar Epithelium.—Very thick sections of 30 to 40 μ are necessary. This is because the alveolar epithelial cells can only be satisfactorily seen by examining them "en face" (not in profile). It is possible, I find, to stain the alveolar walls with Heidenhain's iron-hæmatoxylin in sections of 40 μ in thickness, and such preparations are far more instructive than sections of 5 or 10 μ. The best way to demonstrate the cells and squames of the alveolar epithelium is by impregnation with silver nitrate. The following technique gives good results :

1. Inject the lungs until moderately distended with a 1 or 2 per cent. solution of silver nitrate. Tie off the trachea and place the lungs and heart in a large volume of the same

solution. Leave therein for twenty-four hours. Then slice up the lungs and place the fragments in a fresh change of silver nitrate. Leave therein for another twenty-four to forty-eight hours.

2. Wash in many changes of distilled water. Do this in the light, in a large capsule, on a piece of white paper. Prolong this treatment for twelve to twenty-four hours. The pieces of tissue should go quite brown.

3. Dehydrate, clear, and embed in paraffin.

4. Make very thick sections (40 to 50 μ).

Counter-staining lightly in hæmatoxylin, methylene-blue, or safranin is optional. The sections can be mounted in balsam.

It is a good plan, after removal of the paraffin, to examine the sections with the microscope. If the outlines of the alveolar epithelium are well shown, place the sections in 5 per cent. sodium-thiosulphate (" Hypo ") solution to remove any unreduced silver. If the impregnation is not sufficient, mount direct in balsam, and expose the slide to the light on a piece of white paper. Either sunlight or a half-watt electric lamp will reduce the silver.

The impregnation is usually unequal and capricious—as with any impregnation technique. Alteration of the duration and strength of the silver bath may be necessary.

LYMPH GLANDS

(See SPLEEN, p. 293)

MAMMARY GLAND

Standard methods of fixation and staining may be used.

To demonstrate fat droplets, in the alveolar cavities and in the cells in the active gland, fix thin pieces in Flemming's fluid. Dehydrate, clear, and embed in paraffin, reducing the treatment with high-grade alcohol, xylol, and paraffin as much as is possible. Sections may be stained with safranin, safranin and lichtgrün, or eosin-methylene-blue.

Or frozen sections may be made, after fixation in formol, and stained with Scharlach R or Sudan III. See the methods for the detection of fat (p. 169) in this connection.

Mitochondria and Golgi elements are shown by the standard methods.

MITOCHONDRIA

Suitable methods (already described) are :

Heidenhain's Iron-hæmatoxylin after appropriate fixation (formol ; Regaud's fluid ; Zenker-formol ; Flemming without acetic acid).

The Kull Technique, p. 140.

Bensley's acid fuchsin-methyl-green stain, p. 244.

For Vital Staining, the Janus green method, p. 194.

MUSCLE

Striated Muscle. — Owing to the extreme contractility of living muscle, better micro-anatomical pictures are obtained by fixing pieces of muscle taken from the cadaver than during life. Such points as the insertions of muscles are apt partly to tear away if the contractile and irritable muscle is brought direct into the fixative. Consequently, for purposes of gross microscopic anatomy, the best moment to excise and fix such muscles is during rigor mortis.

Note, however, that for cytological purposes muscle must be fixed immediately after death, since material, as ordinarily obtainable from the post-mortem room, is useless for observations on such delicate elements as myofibrils and mitochondria.

Non-Striated Muscle, such as surrounds the hollow viscera, is best fixed by injecting the fixative into the cavity bounded by the muscle, as described on p. 200.

Cardiac Muscle.—In the case of small mammals the entire heart can be fixed ; or it may be bisected. For larger hearts, segments of requisite size should be cut out.

FIXATION.—Suitable fixatives for general work are :

Formol of 4 to 5 per cent. in 0·9 per cent. sodium chloride
Regaud's fluid.
Mercuric chloride in saturated aqueous solution.
Mercuric chloride-formol.
Flemming's fluid (for very small fragments).

M. HEIDENHAIN'S METHOD OF FIXATION.—Much of this

author's work on the myofibrils and intimate structure of the muscle fibre has been done by the following method :

1. Excise strips of fresh muscle and place them in gauze moistened in 0·9 per cent. sodium chloride until they have cooled and lost their contractility.

2. Pin them out flat with hedgehog quills on a cork which has been soaked in paraffin wax. The paraffin prevents extraction of the tannin from the cork.

3. Fix for twenty-four hours in a 5 per cent. aqueous solution of trichloracetic acid.

4. Transfer the muscle to several changes of 96 per cent. alcohol to extract the acid.

5. Complete dehydration in absolute alcohol ; clear and embed in paraffin.

NOTE.—You must at all costs avoid placing the tissues in water after they have been treated with the trichloracetic acid. This is essential if swelling of the collagen fibres is to be avoided.

EMBEDDING.—For micro-anatomical work, in which large sections and the minimum of tissue shrinkage are required, celloidin should be used. Objects such as the junction of muscle and tendon are too hard after inclusion in paraffin for good sections to be made.

For observations on the finer structure of muscle, thin sections are required ; hence inclusion in paraffin is necessary. The duration of the clearing and embedding processes must be as short as possible. Vacuum embedding (p. 55) is very useful.

Frozen sections are only made when speed is essential or for observations on the fat content of muscle. For the latter, the method of gelatine embedding (see p. 87) should be of use, as frozen sections of muscle are apt to dissociate during manipulation.

STAINING.—General histological methods can be used. For the finer details Heidenhain's iron-hæmatoxylin is unequalled. The mitochondria can be stained by this stain after fixation in Regaud's fluid, and also by the Regaud-Kull technique. The Golgi elements, and the perinuclear band, are impregnated by Cajal's uranium nitrate-formol method.

For nerve fibres in muscle, see p. 272.

For nerve endings in muscle, see p. 275.

NAIL

(See SKIN, p. 286)

NERVOUS SYSTEM

Introductory

The methods for the microscopic study of the nervous system are admittedly the most difficult and complex as yet evolved in Histology. The reasons for this are as follows :

1. *The physico-chemical properties of nervous tissue are widely different from those of other tissues.*

2. *The structural complexity of the nervous system render imperative the use of very delicate methods for the differential staining of its various elements.* A very simple observation will illustrate this point : take a piece of any organ (*e.g.* kidney, liver, or ovary), fix in any standard fixative, make sections of it, and mount the sections unstained. (A mounting medium of low refractive index, such as glycerine jelly, is preferable.) Next, treat in a similar way a section of spinal cord or cerebral cortex. On examination with the microscope you will find that whereas the structure of the first specimen can be fairly accurately defined, that of the section of Central Nervous System will be far from clear. In a section of cerebral cortex, for instance, the outlines of the nerve cells and their nuclei may be seen The white matter can be distinguished from the grey. Here and there, nuclei other than those of nerve cells can be defined. These are the nuclei of neuroglia. And, finally, capillary blood vessels and the connective tissue of the sulci may be made out. The axones and dendrites, the neuroglia fibres and cell bodies—not to mention the intracellular components of the nerve cells —are invisible. In fact, this highly organised and complex nexus is merely represented by the rather indefinite substance between the cells of the cortex. Apply a standard staining method, such as hæmatoxylin and eosin, and you will be able but little—if at all—to resolve further the structure of the nerve centres.

It follows from this that in no branch of histological endeavour are the methods of staining and of impregnation so

SCHEME OF METHODS FOR GROUPED IN RELATION TO THE

METHODS FOR :—

CENTRAL NERVOUS SYSTEM.

- Methods demonstrating structures inside cell bodies and their processes—*i.e.* Cytological Methods.
  - **Nissl Granules:** Methylene-Blue (also Toluidin-Blue or Thionin).
  - **Golgi Apparatus:** Methods of Cajal, Da Fano, and Golgi.
  - **Mitochondria:** Methods of Kull and Heidenhain's Iron-Hæmatoxylin after appropriate fixation. Bensley's Method.
  - **Neurofibrils:** Methods of Cajal and Bielschowsky.
- Methods showing relations between cells and their processes—*i.e.* Micro-anatomical Methods.
  - **Cell Bodies, Axones, and Dendrites:** Methods of Golgi, Cajal, and Bielschowsky.
  - *A.* **Myelinated Fibres:** Methods of Weigert (and its modifications), Kultschitzky, Iron-Hæmatoxylin of Nageotte and of Heidenhain.
  - *B.* **Degenerated Fibres:** Marchi's Method; Scharlach R; Sudan III.
  - **Non-Myelinated Fibres:** Methods of Ranson, Bielschowsky.
  - **Neuroglia Cells and Fibres:**
    Weigert's Method.
    Methods of the Spanish School (Cajal, Rio Hortega).
    Alzheimer's Methods.
    Lhermitte's Method.
    Anglade and Morel's Method.

THE NERVOUS SYSTEM, STRUCTURES STAINED BY THEM.

METHODS FOR :—

- PERIPHERAL NERVOUS SYSTEM.
  - Nerve Fibres.
    - Myelinated.
      - Methods of Weigert (and modifications).
      - Methods of Marchi.
      - „ Bielschowsky.
      - Methylene-Blue (Intravitam) Technique.
    - Non-Myelinated.
      - Methods of Bielschowsky.
      - Methods of Ranson.
      - „ Ranvier (Gold Chloride).
      - Methylene-Blue (Intravitam) Technique.
  - Nerve Endings . . . .
    - Bielschowsky.
    - Ranvier (Gold Chloride).
    - Methylene-Blue (Intravitam) Technique.

important as in the case of the central nervous system. And it may be said—a retrospective survey of the literature will confirm this point—that the knowledge of the nervous system, functional as well as micro-anatomical, has increased largely as the outcome of improved histological methods. The neurone theory itself is founded on a microscopic conception which was only verified when sufficiently precise methods had been evolved.

3. *Methods for the nervous system are still somewhat in a state of flux.* The recent technical contributions of the Spanish school make it clear that there is still plenty of room for new methods and for fresh discoveries accruing from their use. This is in contrast with the now largely stereotyped techniques for other tissues.

4. *It is, up to date, impossible to show, by one and the same method, anything approaching the totality of the elements of the C.N.S.* Thus, to demonstrate nerve cells and their processes, methods have to be used which render it impossible to see the cytological details of the cells themselves. Or, if a specific method for neuroglia be employed, the processes of nerve cells will not be suitably stained. Or again, when investigating certain components of nerve cells, such as Nissl granules, mitochondria, or Golgi bodies, the courses of axones and dendrites cannot be traced. It follows from this that the histology of the nervous system is the outcome of the integration of the appearances furnished by a number of different methods, each of which tends to pick out, and render capable of study, certain elements only of that vast mass of cells, cell constituents, cell processes, and fibres which goes to make up the most complex cell organisation in the body.

5. *The complexity and the intervention, of factors as yet unknown, makes it often difficult to stain particular elements of the nervous system in the same way on different occasions.*

The chances of introducing variants into the technique are greater here than elsewhere. This applies especially to the metallic impregnation methods, the use of which is still largely empirical. Whence the necessity for the examination of a large number of preparations, when certain methods are used, before a true idea of the elements under study can be obtained.

Some idea of the methods used in neurological histology

may be gained from the foregoing scheme, though it must be remembered that it only gives a selection of the most important methods now known, which are classified in relation to the structures they demonstrate.

ARTEFACTS DUE TO TECHNIQUE

Probably nowhere in histological technique does the following question assume such importance as in the case of the C.N.S. : "Are the appearances given by the method employed a reliable simulacrum of the living tissue or not ? and, if not, what must be added to, or taken away from, the images furnished in order to arrive at a tolerably clear idea of the structure of the tissues during life ? "

The problem of the effects of fixation on the intimate structure of protoplasm having been already discussed (see Chapter IV.), only a very brief précis on the validity of the various structures which have been described in the nervous system is given here.

The Nerve Cells, and their Processes (axones and dendrites) certainly exist in the living organism in much the same disposition as they are seen in suitably treated microscopic preparations.

The Neuroglial Complex of Cells, Processes, and Fibres.— The same is applicable to this.

Mitochondria, Pigment, and various Lipoidal Granules are visible intravitam, and are morphologically the same after appropriate treatment.

The Golgi Network, in the light of all recent researches, as seen in suitably prepared sections, probably represents with fair topographic accuracy a similar system existent during life in nerve cells. But, as pointed out by Cowdry, the solid appearance of the Golgi bodies in stained specimens of mammalian cells is an artefact, in view of the fluidity of their protoplasm. It has also been suggested that the Golgi network may be the stained counterpart of a system of vacuoles (Parat).

Holmgren Trophospongium. — The significance of this is debatable. All that can be said is that Penfield has conclusively shown that the Holmgren canaliculi are not the same as the Golgi elements in the case of nerve cells.

Nissl Granules. — The observations of Mott on the living nerve cell make it very doubtful whether these, as seen in fixed and stained specimens, exist as such in the living cell. This, of course, does not imply that the study of these bodies is a useless vagary. The mere fact that the normal nerve cell can be made to show Nissl granules, and that the other cells of the body cannot, shows at least that a substance or substances, capable of forming characteristic precipitation products on being fixed, are exclusively present in nerve cells. Further, the fact that Nissl granules decrease and finally disappear in nerve cells which have been fatigued, or of which the axones have been severed or damaged, forms a valuable means of gauging, on a microscopic basis, the physiological condition of such cells.

Neurofibrils.—It is significant that these structures cannot be seen in living nerve cells (Mott ; Lewis ; Matsumoto) with either direct or dark-ground illumination. Even if their refractive index were too akin to that of the protoplasm of the cell to be seen by direct illumination, one would expect that the dark ground—which even reveals colloidal granules—would show up some structure comparable to these fibrils. There is another observation, of a physiological nature, which further invalidates the neurofibrillar conception. Carlson, in the course of stimulation experiments on the pedal nerves of the slug, noted that while the rate of conduction was slower in the artificially stretched nerve, its excitability was not affected. This can only be assumed by regarding the axis cylinder as a liquid, since the fine neurofibrils, if stretched to twice their length, as was the case, could hardly conduct in an unaltered manner. Consequently the excitability of the nerve would be diminished—which it is not. Neurofibrils, in brief, appear to be due to precipitation of the protoplasm of the nerve cell and its processes along certain lines of action. But it must be remembered that if the famous Bielschowsky technique for neurofibrils is open to criticism in so far as the validity of the images of these fibrils is concerned, it remains one of the most reliable methods for mapping out the distribution of axones, dendrites, and end organs. And, finally, the study of neurofibrils under different

physiological conditions is of value, since changes in their size and distribution have been noted. They therefore appear to be of some value as an index of the activity of the cell, although there is no reason to believe in their existence, as neurofibrils, in the living nerve cell and in its processes.

Neurokeratin Sheath of Medullated Nerves.—This is probably a gross precipitation artefact (Cajal).

ARTEFACT DUE TO POST-MORTEM CHANGES

The C.N.S. is particularly prone to autolytic changes after death. It may generally be said that freshness of the tissues is more important for the cytological and neuroglial methods than for the methods of Weigert, Marchi, and Bielschowsky. (This remark only applies to the latter method when it is used micro-anatomically—*i.e.* for the processes of nerve cells. For work on neurofibrils, by this or by any other method, the tissues must be perfectly fresh.) In all histopathological work on the nervous system, every effort should be made to perform the autopsy within two to three hours of death, or, failing this, to proceed with Pierre Marie's "Formolage *in situ*" (see under) immediately after the decease.

Neuroglia are especially sensitive to post-mortem change; the average autopsy, performed some twenty-four hours after death, is useless from the standpoint of specific neuroglial staining. These delicate reactions are not furnished by material which is more than a few hours post-mortem. In cases which have died with hyperpyrexia, autolysis of the neuroglia occurs even earlier. Though such changes are slowed down by low temperature, neuroglia appear to suffer fairly rapidly even if the cadaver be placed in a cold storage chamber. Thus, Cajal has reported autolysis of the neuroglia in the case of a man who died from severance of the femoral artery, and whose body was kept at plus 4° C. for forty-eight hours.

End organs should always be fixed immediately after excision from the living or just dead subject.

Pierre Marie's Method of "Formolage" *in situ*.—This simple expedient makes it possible to obtain well-preserved

material even when the autopsy is made twenty-four hours or longer after death.

The following are requisite :

A stout cannula and trochar.

A syringe of 200 c.c. capacity.

A 5 or 10 per cent. solution of formol.

You proceed thus :

(1) Immediately, or as soon as possible, after the subject's death, insert trochar and cannula into one of the nostrils ; push the trochar upwards past the inferior turbinals (which will hold it in place) and continue until the resistance of the ethmoid is felt.

(2) Holding the cannula in the left hand, sharply tap it with the right. You will feel as soon as the cribiform plate has been pierced, and the arachnoid space has been entered. Then remove the trochar.

(3) With a large syringe inject, *very slowly*, 150 to 200 c.c. of 5 or 10 per cent. solution of formol.

The autopsy is subsequently made in the usual manner.

The *advantages* of this method are that post-mortem changes in both brain and spinal cord are avoided, and that removal and macroscopic section of the brain and spinal cord is easier than in the autolysed and softened tissues usually encountered on the post-mortem table.

The *disadvantages* are that certain methods (*e.g.* of Nissl) demand special fixatives, and that the colour of the brain and spinal cord is altered. Approximately the original colour can be restored, however, by placing pieces of the tissue in alcohol of 60 or 70 per cent.

Application of the Various Histological Methods.—These are obviously used in relation to those elements of the nervous system which it is intended to study. Owing to the variable results often furnished by any given method, it is a good plan to control such results by applying more than a single method.

In Histo-Pathological Neurology the following methods are specially useful :

For a general survey of the tissues and of inflammatory or other lesions :

Hæmatoxylin (of Delafield or Ehrlich) and eosin.

Hæmatoxylin and van Gieson.

Heidenhain's iron-hæmatoxylin.

For the study of the nerve cells, with special reference to the Nissl granules :

Methylene-blue (or a substitute such as toluidin-blue or thionin).

For the study of the processes of nerve cells :

Bielschowsky's silver impregnation method.

For the study of degenerative changes in the tracts of the nervous system :

If long standing : the Weigert technique, or one of its modifications.

If recent : the Marchi technique, or one of the specific fat stains, such as Sudan III. or Scharlach R.

NOTE.—The Weigert method (and its modifications) give a *negative* stain of degenerated myelin, which is unstained, in contrast to normal myelin, which is *positively* stained blue to black. The Marchi (and specific fat) stains give a *positive* impregnation of degenerated myelin (black with Marchi, yellow with Sudan III., red with Scharlach R), while normal myelin is *unstained*. But since the myelin of degenerated fibres is progressively removed by phagocytes, the Marchi (and specific fat) techniques are only applicable for from ten days to a maximum of two months after the onset of degeneration. *In practice, the useful application of these methods is restricted to degenerations of medullated fibres, the age of which is about one month. For older degenerations the Weigert method* (or one of its modifications) *must be employed.* For further discussion of these methods, see the sections devoted to them.

For the study of Neuroglia :

The methods of the Spanish school (Cajal and Rio Hortega).

Lhermitte's method.

Weigert's method.

Mallory's method.

Alzheimer's method.

CENTRAL NERVOUS SYSTEM

CYTOLOGICAL METHODS

Nissl Granules

The significance of these is discussed on p. 238. It must be noted that the same technique—particularly for fixation—

must always be employed when comparative results are desired. Further, in order to estimate the amount of Nissl substance all sections should be of the same thickness (under 10 μ). Fixation in 95 or 96 per cent. alcohol gives the sharpest images of Nissl substance.

Nissl's original method is long and complicated. It may advantageously be replaced by the modification given beneath.

Modified Nissl Staining (of Roussy and Lhermitte)

1. Fix pieces of tissue not thicker than 1 cm. in 96 per cent. alcohol for two to four days.
2. Make sections without embedding [1] or embed in celloidin. It is also possible to obtain excellent preparations—in spite of all that has been said to the contrary—after inclusion in paraffin.
3. Stain for six hours or more at room temperature in one of the following solutions :
   1 per cent. toluidin blue ;
   1 per cent. thionin ;
   Grübler's polychrome-methylene-blue.
   (Borrel's methylene-blue, diluted in the proportion of 1 to 5 (of distilled water) gives excellent results.)
4. Rinse rapidly in water.
5. Dehydrate for one minute in 90 per cent. alcohol.
6. Differentiate, controlling the extraction of the stain with the microscope, in *Gothard's differentiator :*

Pure creosote	50 c.c.
Cajuput oil	40 c.c.
Xylol	50 c.c.
Absolute alcohol	150 c.c.

7. Rinse the sections in absolute alcohol.
8. Clear in xylol.
9. Mount in neutral balsam or dammar.

[1] To do this, blot the tissue with filter paper and mount on a vulcanite fibre block smeared with thick celloidin (or collodion) solution. Harden the union in 80 or 90 per cent. alcohol. Then cut on a sliding microtome, keeping the knife flooded with alcohol, as for " wet " celloidin sections.

Nissl bodies, blue against the unstained cytoplasmic background ; nuclei, nearly colourless—except for the nucleoli.

NOTE.—This method is simpler than the original Nissl technique, and can be used for both celloidin and paraffin sections. The advantage of the latter is the ease with which thin serial sections may be made.

EOSIN-METHYLENE-BLUE (SEE TYPE METHODS, p. 133)

This gives very good pictures of Nissl granules after fixation in alcohol or in Zenker's fluid. It is advisable to differentiate the stain in colophonium-alcohol (see p. 133) as advised by Mallory and Wright.

Golgi Network (=Golgi Reticulum ; Golgi Bodies, etc.)

CAJAL'S URANIUM NITRATE-FORMOL METHOD

Both this and Da Fano's modification give excellent results with nerve cells. These methods are described on p. 142. Freshness of the tissues is important, as autolysis of the Golgi apparatus occurs fairly rapidly after death, beginning, according to Cajal, some two hours after death in man.

For experimental investigations Penfield reports perfect fixation by the injection method. This is done as follows :

A laminectomy in the lumbar region is performed in the anæsthetised animal and the membranes are exposed.[1] A needle is passed into the subarachnoid space and is directed upwards a short way along the cord. The Cajal fixative is injected under a gravity pressure of about 750 mm. (of water). The heart-action ceases after about a minute, but the injection is continued for twenty hours. The tissues are then placed directly in the silver bath.

GOLGI'S ORIGINAL METHOD

Fix thin pieces of tissue for three to twelve hours in the following fluid :

Saturated aqueous solution of arsenious acid 20 per cent. formalin in distilled water . 96 per cent. alcohol.	Equal parts.

[1] A vivisection licence is necessary to perform this operation in Great Britain.

2. Rinse the tissues rapidly in distilled water.

3. Impregnate with 1 per cent. silver nitrate for several hours or days. (The best plan is to take pieces of the tissue out of the silver bath every six hours or so.)

4. Reduce in the developer advocated by Cajal for his method for impregnating the Golgi apparatus (see p. 114).

Subsequent treatment as for the Cajal method (p. 143).

Golgi reticulum, black.

For the Mann-Kopsch technique, see Gatenby's article in the *Microtomist's Vade Mecum*, p. 328.

The *relations between the Golgi reticulum and the Holmgren canaliculi* of nerve cells are well shown by Penfield's ingenious technique, which consists in impregnating the tissue by the Cajal method and making camera lucida drawings of the Golgi apparatus in certain cells. The cover-slip is then removed with xylol, and the sections passed into the iron-alum solution of Heidenhain, in which they remain for twelve to twenty-four hours. All the silver impregnation is dissolved by this, after which the sections are stained in hæmatoxylin, differentiated in iron alum and carefully passed through upgraded alcohols to xylol. They are then mounted in balsam. Care must be taken during differentiation to ensure that the Holmgren canaliculi, but not the ground cytoplasm, are destained. Canaliculi clear, ground cytoplasm in tones of grey.

Mitochondria

Standard methods may be used. Mitochondria are stained by *Heidenhain's iron-hæmatoxylin* after fixation in Regaud's formol-bichromate (see p. 40), but they are not tinctorially differentiated from the Nissl granules.

Kull's technique (see p. 140) after Regaud's fluid is good, and so is Bensley's methyl-green-acid fuchsin, which is used as follows :

1. Fix in Bensley's fluid for twenty-four hours :

2 per cent. osmic acid . . .	2 c.c.
2·5 per cent. potassium bichromate .	8 c.c.
Glacial acetic acid	1 drop.

Any other chrome-osmium fixative, or Regaud's fluid, may be used instead.

2. Wash for twelve hours in running water.

3. Dehydrate, clear, and embed in paraffin.

4. Make thin sections (not more than 5 μ thick).

5. Take sections on the slide down to water.

6. Treat with 1 per cent. potassium permanganate for thirty to sixty seconds.

7. Treat with 5 per cent. oxalic acid for thirty to sixty seconds.

8. Rinse in several changes of distilled water.

9. Stain in hot acid fuchsin (formula, p. 110) for about six minutes. (I prefer to heat the fuchsin in a test-tube till bubbles begin to come off—*i.e.* to about 60° C.—and then pour the hot stain on the sections.)

10. Flush away all precipitate of fuchsin in a stream of distilled water.

11. Pour on a 1 per cent. aqueous solution of methyl-green : the staining time varies from five seconds upwards and can only be ascertained by trial.

12. Dehydrate rapidly in absolute alcohol.

13. Clear in xylol.

14. Mount in balsam or dammar.

Mitochondria, bright red ; nuclei, green ; red blood corpuscles, dark red. The stain fades in course of time, particularly if the sections are not kept in the dark.

Notes.—(1) Differentiation in the methyl-green is the crucial operation. In under-differentiated sections the cytoplasm is homogeneously stained dark red, in over-differentiated ones the methyl-green substitutes itself in the place of the acid fuchsin.

(2) It is a good plan to proceed as far as stage 13, and then examine with the staining microscope. If the sections be insufficiently stained you can then pass down either to the methyl-green (if the fuchsin be still too dark) or to the fuchsin (if insufficiently stained by this).

(3) The preliminary mordanting with potassium permanganate and oxalic acid can often be omitted.

Neurofibrils

1. The validity of the images furnished of these structures is discussed on p. 238. But the following methods are also very valuable for mapping out the relations between the processes of the nerve cells.

The standard methods for vertebrates are those of Cajal and Bielschowsky.

Cajal's Technique

Cajal himself has introduced many modifications of his original method, each variant being especially suitable for the impregnation of neurofibrils in certain regions of the central nervous system in various animals and developmental stages. His most important modifications are summarised beneath.

The general principle in all is that of impregnation of the elements to be demonstrated with silver nitrate, which is subsequently reduced to a compound not yet determined, by means of a modified photographic developer.

Note.—These methods require, during the silver bath, a temperature of 28° to 35° C. If an incubator, kept at such temperatures, be not available, the tissues may be placed on the top of the embedding oven or any other simple expedient adopted.

Method I.—*Especially suited for the medulla, bulb, cerebellum, and spinal ganglia of embryos and recently born animals; also cerebellum and cerebrum of adults.*

1. Place pieces of tissue of 3 mm. thickness in a 1·5 per cent. solution of silver nitrate at 35° C. for three to five days.

As with all impregnation methods, the volume of the metallic salt solution should be large—100 c.c. for quite small fragments of nervous system. Better results are sometimes obtained by the addition of 10 to 20 per cent. of absolute alcohol to the silver bath.

2. Wash the tissues rapidly in distilled water.

3. Treat for twenty-four hours in the following reducing fluid:

Pyrogallic acid or hydroquinone . .	1 to 2 grms.
40 per cent. formol	5 to 10 c.c.
Distilled water	100 c.c.

4. Rinse rapidly in distilled water and embed in celloidin or paraffin.

In sections—preferably of moderate thickness (10 μ)—neurofibrils are impregnated black by this and the succeeding methods. The outer layers of the tissues are usually quite black owing to over-impregnation.

METHOD II.—*For cerebellum and cerebrum; also for nerve endings of Pacini, Meissner, Krause, and Meckel. Both medullated and non-medullated axis cylinders are well shown.*

1. Fix tissues for twenty-four hours in 40 per cent. to absolute alcohol. The addition of veronal in the proportion of 2 per cent. to the alcohol makes for a better impregnation.

2. Treat with 1·5 silver nitrate at 28° to 35° C. for five to seven days. (Five days is sufficient after the veronal-alcohol fixation.)

Subsequent treatment as for Method I.

METHOD III.—*Especially good for spinal cord and spinal ganglia of young animals.* By reducing the ammonia to 1 to 3 drops, peripheral nerve endings may be impregnated.

1. Fixation for twenty-four hours in :

Absolute alcohol . . .	50 c.c.
Ammonia	5 to 10 drops.

Subsequent treatment as for Methods I. and II.

METHOD IV.—*Cajal's favourite method for the study of degenerative and regenerative processes in the central nervous system.*

1. Fix for twenty-four hours in—

Pure pyridin .	40 c.c.	OR	Pure pyridin .	Equal parts.
96 per cent. alcohol	20 c.c.		Distilled water	

2. Wash for six hours in running water.

3. Place in 96 per cent. alcohol for six to twelve hours.

4. Rinse in distilled water to extract the alcohol.

(This is effected soon after the tissues sink.)

5. Impregnate in 1·5 per cent. silver nitrate for four to five days at 35° C., or for three days at 40° C.

Reduction and further treatment (paraffin or celloidin sections) as for preceding methods.

METHOD V.—*Excellent for motor end-plates, and also for pericellular arborisations of the central nervous system.*

1. Fix tissues for twenty-four hours in a 10 per cent. aqueous solution of chloral hydrate.

2. Rinse in distilled water.

3. Transfer to ammoniacal alcohol (ammonia, 4 drops; 96 per cent. alcohol, 50 c.c.) for twenty-four hours.

Impregnation and other operations as for preceding methods. (Four to five days at 35° to 38° C. in 1·5 per cent. silver nitrate.)

The Bielschowsky Technique

This technique gives precise and constant results with the central nervous system of man. It also has the advantage (for the pathologist) of being applicable to formol-fixed material. Da Fano reports successful Bielschowsky impregnations with brains which had lain in formol for over eleven years. And, finally, the method can be applied either to frozen sections or to pieces of tissue which, after impregnation, may be embedded in paraffin.

The general principle of the method seems to be as follows:

The addition of ammonia to a solution of silver nitrate in a test-tube produces a precipitate of silver hydroxide [$Ag(OH)_2$], which is soluble in an excess of ammonia, when a solution of ammoniacal silver hydroxide [$Ag(NH_3)_2OH$] is formed. Now, if a reducing agent, such as formol, be added to this solution, a deposit of metallic silver is thrown down on the wall of the test-tube.

This reaction occurs in tissues treated by the Bielschowsky method, but the reason for the selectivity of the silver deposit for nerve cells and their processes is unknown.

Note.—The glass vessels in which impregnation and reduction are effected must be *absolutely clean*. Soaking in the potassium bichromate-sulphuric acid mixture followed by distilled water and drying in an oven will ensure this. Manipulate the sections with a glass rod and rinse it in distilled water between the various operations.

Method A.—*Impregnation of Sections.*

1. Fix pieces of tissue up to 2 mm. in thickness in 4 per cent. formol. If the fragments are thick, fixation should be prolonged for seven to ten days, though thin slices are fixed in twenty-four to forty-eight hours.

2. Place in water for one hour.

3. Make frozen sections. For neurofibrils they should be 5 μ in thickness, for micro-anatomical observations, about 10 μ.

4. Place the sections in distilled water for one hour.

5. Transfer the sections to a capsule or deep Petri dish of 2 to 4 per cent. silver nitrate (the latter is best for human material). Keep in the dark for twenty-four to forty-eight hours.

6. Rinse rapidly in distilled water.

7. Treat with the ammoniacal silver nitrate solution (prepared just before use) for three to ten minutes.

To prepare this, add 6 drops of a 40 per cent. solution of sodium hydrate to 5 c.c. of a 20 per cent. solution of silver nitrate. A brown precipitate is formed. Now cautiously add strong ammonia till the precipitate is just (or, better, nearly) dissolved. Usually about 5 c.c. of concentrated ammonia are required. Make up to 25 c.c. with distilled water and filter through a filter paper moistened with distilled water.

When the sections have turned brown transfer them to :

8. Distilled water, in which they are rapidly washed.

9. Reduce the silver in 4 per cent. formol for ten minutes. This solution should not be acid, but (preferably) slightly alkaline. This is easily attained by diluting neutral formalin with tap water.

10. Wash the sections for at least fifteen minutes in distilled water.

11. Tone in an 0·2 per cent. solution of gold chloride until the sections are grey.

12. Rinse rapidly in distilled water.

13. Remove any unreduced silver by treatment for one minute with sodium thiosulphate of 4 or 5 per cent.

14. Wash in distilled water. (Counter-staining in neutral red or safranin is optional after this stage.)

15. Dehydrate in ascending grades of alcohol.

16. Clear in xylol.

17. Mount in dammar or balsam.

Neurofibrils, axis cylinders, and dendrites are impregnated an intense black. Collagen and neuroglia fibres, sometimes impregnated.

Notes.—(1) If the toning bath be slightly acidified with acetic acid, the background of the sections is a faint purple ; if neutralised with lithium carbonate it is grey.

(2) If the sections are placed for twenty-four to forty-eight hours in pure pyridin between stages 3 and 4, the connective tissue, and especially the neuroglia, have less tendency to stain. The pyridin must be carefully removed by distilled water before the sections are placed in the silver bath.

Method B.—*Impregnation of Tissues in Bulk followed by Inclusion in Paraffin.*

The results are not as constant as by the first method.

1. Fix in 6 to 8 per cent. formol for at least a week.
2. Wash in running water for a few hours.
3. Post-fixation in pyridin for two days.
4. Place in running water for twenty-four hours.
5. Constant changes of distilled water for twenty-four hours.
6. Impregnation in the dark with a 2·5 to 3 per cent. solution of silver nitrate for four to five days.
7. Rinse rapidly in distilled water.
8. Treat for four to five hours in a dilute ammoniacal-silver nitrate bath prepared thus :

Add 3 drops of a 40 per cent. solution of sodium hydrate to 4 c.c. of a 10 per cent. solution of silver nitrate. Add concentrated ammonia till the precipitate is almost (or just) dissolved. Make up to 20 c.c. with distilled water.

9. Rinse the tissues in distilled water for one-half to one minute.
10. Treat the pieces of tissue with 8 per cent. formol for twelve hours.
11. Wash rapidly in distilled water.
12. Dehydrate, clear, and embed in paraffin.
13. Make sections 10 μ thick and mount on slides.
14. After the usual treatment with xylol and descending grades of alcohol—
15. Tone for five to ten minutes in :

Distilled water	20 c.c.
2 per cent. gold chloride . .	3 drops.

16. Rinse in distilled water.

17. Treat with 4 or 5 per cent. sodium thiosulphate ("Hypo") for a few minutes.

18. Place in distilled water for half an hour. (Staining with neutral red or safranin is optional after this.)

19. Dehydrate in ascending grades of alcohol.

20. Clear in xylol.

21. Mount in dammar or balsam.

MICRO-ANATOMICAL METHODS

Axons and Dendrites

A. **The Golgi Methods.**—The basis of the original methods is a treatment of pieces of central nervous system with potassium bichromate (either alone or combined with osmic acid), this being followed by impregnation with silver nitrate. The nature of the impregnation is not clear.

In the case of the "slow" Golgi method (prolonged fixation in potassium bichromate followed by treatment with silver nitrate), there is little doubt (A. Hill) that the impregnation is a reduced silver salt, though the composition of this has not been established. It is not a metallic oxide, nor is it reduced as far as in photography, since the Golgi deposit is soluble in sodium thiosulphate and in dilute ammonia. Nor can it be silver chromate, since it is black.

As to the nature of the deposit formed by the Golgi-Cox method, nothing definite is known beyond that it contains mercury and is white until blackened by the action of ammonia or sodium carbonate.

The characteristics of these methods are as follows :

1. While they exhibit an undoubted affinity for the processes and bodies of nerve cells and (usually) of neuroglia also, only a few cells are picked out by them in a given preparation. Even when the cells and processes are of the same type, and lie side by side, only some of them are demonstrated. Why this should be so is not known, but it has the practical advantage of enabling the relations of cells to each other to be made out with the microscope. If *all* the cells and *all* their processes were shown in a given section of (say) cerebral cortex, it would be difficult (if not impossible) to follow their courses.

2. The above peculiarity, while rendering the Golgi methods very valuable in the study of the normal nerve centres, is a distinct disadvantage for histo-pathological studies. For here the destruction or degeneration of *certain* nerve cells is most important; consequently the Golgi methods, which only pick out a few cells and processes, are not reliable for such observations. Whence the preference of pathological neurologists for the Bielschowsky technique.

3. The Golgi methods are capricious, and it is difficult to get the same results in different specimens—even of the same tissue.

Notwithstanding the above, the methods of the Italian savant are invaluable for the study of the central nervous system; the clarity of the images furnished by them in successful preparations is extraordinary, and their introduction led to epoch-making discoveries.

Three processes have been elaborated by Golgi, known as the "Slow," the "Rapid," and the "Mixed." There is also an important modification of the latter, due to Cox. This process is generally known as the Golgi-Cox method. Only the rapid method of Golgi and the Golgi-Cox technique are given here. The latter is particularly recommended: in my hands it has proved the least capricious of the Golgi group.

Freshness of Tissues.—Although these methods may succeed with ordinary autopsy material, the images furnished by such are naturally open to discussion.

The Golgi "Rapid" Method.—The advantage of this method is that sections may be made in a week or ten days; its disadvantage is that it is only applicable to very small pieces of tissue.

1. Place the tissues in the following mixture for two to seven days:

2·5 to 3 per cent. potassium bichromate	4 parts.
1 per cent. osmic acid	1 part.

From the third up to the seventh day, transfer a piece or pieces to:

2. 0·75 per cent. silver nitrate for twenty-four to forty-eight hours. A precipitate is formed, and the bath should be renewed several times in the course of forty-eight hours. Tissues may remain without harm for days in the silver solution.

3. Place the tissues in 80 per cent. alcohol to remove any unreduced silver. The alcohol must be changed repeatedly until no longer yellow.

4. Make sections by one of the following methods :

(*a*) *Cut them Freehand*—suitable for determining whether the chroming process has been sufficiently prolonged.

(*b*) *Cut Unembedded on the Microtome.*—To do this, stick the block of tissue on to a vulcanite fibre block by wiping it dry ; smear its base with thick celloidin solution, and press it down. Then harden the joint in chloroform vapour, soak in 80 per cent. alcohol for a short time, and cut on the sliding microtome.

(*c*) *Make Frozen Sections.*—The tissues are placed in a 20 per cent. solution of neutral dextrin (G. Mann) after rinsing in water. Impregnation with the dextrin takes one to three days according to the thickness of the blocks. They are then transferred direct to the microtome.

(*d*) *Embed in Celloidin.*—In A. Hill's experience this can be done fairly slowly—*i.e.* up to four days' impregnation in celloidin solution. (See " ' Wet ' Celloidin Process," p. 61.)

(*e*) *Embed rapidly in Paraffin.*—Take thin slices of tissue and dehydrate rapidly (*i.e.* in one to two hours) in absolute alcohol. Transfer to oil of cedar (xylol is said to injure the impregnation) and thence direct to paraffin.

Note.—Thin sections are not desirable as the ramifications, etc., of dendrites cannot be traced in them. Golgi material is generally cut at 20 to 100 μ.

For systematic work, methods (*c*) and (*d*) are the best.

To see if the impregnation is sufficient, use method (*a*).

5. Place the sections, as cut, in absolute alcohol.

Wash them in several changes of this.

6. Clear in creosote for a few minutes.

7. Transfer to turpentine.

Or, in place of 6 and 7, you can clear in oil of cedar.

8. Mount in balsam, *but do not cover the sections.* Sections impregnated by the Golgi method, if mounted between a coverslip and slide, rapidly fade. Therefore one of the following methods must be adopted :

(*a*) Mount in thick xylol dammar (or xylol balsam) and place the slide on the hot plate heated to 40° or 45° C. to harden the mount.

(*b*) Melt a little solid Canada balsam ; warm a slide till it can just be held against the cheek. Then transfer the section to the slide and quickly add the liquid balsam. (It is advantageous to do these operations on a hot plate.) When the section has been orientated, the slide is put aside to cool. If you particularly wish to add a cover-glass, you may do so, as there is less risk, by this method, of the section fading.

Nerve cells and their processes are black ; neuroglia cells and processes (if demonstrated), similarly stained. The impregnation is not transparent, so that the constituents of the cells (*e.g.* nuclei) can not be seen.

Generally the processes of the nerve cells are the first to stain, then the bodies of the cells, and lastly neuroglia cells and their processes. Blood vessels may also be impregnated.

The Golgi-Cox Potassium Bichromate-Mercuric Chloride Method.—This modification of the third Golgi method is very good. Although slow, it is applicable to whole brains of young animals, and to large segments of adult cerebrum, preferably of not more than 1 cm. in thickness.

1. Fix in the Golgi-Cox solution :

5 per cent. potassium bichromate . .	10 parts.
5 per cent. mercuric chloride . . .	10 parts.
5 per cent. potassium chromate . .	8 parts.
Distilled water	15 to 20 parts.

Mann advises that the potassium bichromate and the mercuric chloride solutions be mixed together ; the potassium chromate solution is mixed with the distilled water and then added. After warming the fixative in the incubator, it is ready for use.

Place the tissues, resting on cotton wool, in at least thirty times their bulk of fixative. Change the solution after twenty-four and forty-eight hours' incubation. Then seal the vessel with vaseline and keep it in the incubator at *circa* 37° C. at least one month, and preferably for two months. The tissues must be kept in the dark during this process.

2. Make sections by one of the following methods :

(*a*) Freehand (good for telling if the impregnation is sufficient).

(*b*) With the freezing microtome, as advocated by Mann, after soaking for one to three days in a 20 per cent. solution of neutral dextrin.

(*c*) After embedding in celloidin.

50 to 100 μ is a useful thickness.

3. Blacken the impregnation by placing the sections for about an hour in *either* 5 per cent. sodium carbonate *or* in 5 to 10 per cent. ammonia solution.

4. Wash well in distilled water.

5. Mount as for the other Golgi methods.

If you reduce the impregnation substance (stage 3) the nerve cells and their processes are black. If you omit stage 3 they are white.

NOTE.—The injection of the brain, through the carotid artery, with the fixing fluid is said (A. Hill) to give a more even impregnation. The blood must be washed out first—preferably with 0·9 per cent. sodium chloride to which 1 per cent. of lactic acid has been added. This causes great dilatation of the blood vessels before injection with the fixative. See, in this connection, the technique of injection (p. 188).

B. **The Cajal Methods.**—These are the same as the neurofibril methods described on p. 246. The only point to be noted is that whereas thin (*i.e.* 5 μ) sections are most suitable for observations on neurofibrils, considerably thicker ones (15 to 20 μ) are indicated for the study of the relationships of cell processes.

C. **The Bielschowsky Method.**—This is used as for neurofibrils, but slightly thicker sections are requisite (10 μ or more). The technique is described on p. 248, and is especially suitable for reasons there stated for histo-pathological investigations.

D. **Rio Hortega's Silver Carbonate Method.**—See note on p. 268.

MYELINATED FIBRES

Weigert's Latest (1891) Method. — The principle of this method, according to Wlassak and Mann, is as follows : The cerebrosins and lecithins are constituents of the normal myelin

sheath. When treated with chromium salts — as in the Weigert method—these substances are reduced to chromium dioxide (CrO_2). And it is this latter, which is formed wherever there are normal myelin sheaths, that acts as a mordant towards the subsequent hæmatoxylin stain and forms a lake with it.

A similar principle is presumably involved in the Kultschitzky method for myelin sheaths.

The Weigert technique (and the Pal modification) remain the best methods for demonstrating myelinated fibres. Note that degenerated myelin is unstained by both these. Hence a degenerated tract in a section of spinal cord appears as a white area. Both methods give good results with human and animal material.

Both the Weigert method and Pal's modification have respective advantages and disadvantages, which are summarised on p. 258.

1. Fix pieces of tissue in one of the following fluids :

(*a*) *Formol of* 5 *to* 10 *per cent.*—Material is placed in this for ten days or indefinitely. For general purposes this method of fixation is the most convenient, especially since it allows of " formolage *in situ* " for human subjects.

Note that if you fix in formol, post-fixation by one of the following methods—preferably (*c*)*—is essential :*

(*b*) 5 *per cent. potassium bichromate* in the incubator. This method is very slow and requires weeks. The tissues are sufficiently fixed when the grey matter is stained dark brown and the white matter light brown.

(*c*) *Weigert's Rapid Fixative.*—Tissues not thicker than 1 cm. are fixed, at room temperature, in five to ten days. For fragments from 1·5 to 2 cm. in thickness a fortnight's treatment is necessary.

The composition of this fluid is :

Potassium bichromate . . .	5 grms.
Fluorchrome (Cr_2F_6) . . .	2 grms.
Distilled water	100 c.c.

To prepare this, boil the potassium bichromate in water, add the fluorchrome, then cool and filter.

2. Wash overnight in running water.
3. Embed in celloidin.
4. Make sections 10 to 20 μ in thickness.
5. Place the sections in distilled water.
6. Mordant the sections for twenty-four hours at 37° C. in a bath which is prepared as follows :

Fluorchrome	2·5 grms.
Boiling distilled water	100 c.c.

To which is added—

Glacial acetic acid	5 c.c.
Neutral powdered copper acetate	5 grms.

Then cool to 37° C.

7. Transfer to distilled water.
8. Stain for twenty-four hours in Weigert's iron-hæmatoxylin, which is prepared thus :

Hæmatoxylin	1 grm.	= Solution A.
Absolute alcohol	100 c.c.	
Liquor ferri sesquichlorati (German Pharmacopeia) (= 30 per cent. aqueous solution of ferric chloride)	4 c.c.	= Solution B.
Hydrochloric acid	1 c.c.	
Distilled water	100 c.c.	

Mix equal parts of A and B before use.

9. Wash in several changes of water.
10. Differentiate for fifteen minutes to several hours in :

Borax	2 grms.
Potassium ferricyanide	2·5 grms.
Distilled water	100 c.c.

The fluid may need to be changed if differentiation slows down.

The sections are differentiated when the myelin is black, the grey matter yellowish.

11. Wash in several changes of water.
12. Dehydrate, clear, and mount in dammar or balsam.

Myelinated fibres, black ; recent areas of degeneration, pale grey ; old ones, colourless.

Weigert-Pal Technique

NOTE.—*This method may be used with both paraffin and celloidin material.*

1. Fix in potassium bichromate of 2·5 per cent. for several weeks in the incubator. Or fix in formol, then treat with the potassium bichromate.
2. Dehydrate and embed.
3. Make sections of 10 to 20 μ.
4. Stain in the following lithium carbonate-hæmatoxylin mixture for twenty-four to forty-eight hours :

SOLUTION A

A ripened solution of hæmatoxylin . .	10 grms.
Absolute alcohol	100 c.c.

SOLUTION B

Concentrated aqueous solution of lithium carbonate	7 c.c.
Distilled water	93 c.c.

Mix before use in the proportion of 1 volume of A to 9 volumes of B.

5. Treat with 0·25 per cent. potassium permanganate for one-half to three minutes.
6. Rinse the sections in water.
7. Treat for one-half to three minutes with the following differentiating fluid :

1 per cent. aqueous solution oxalic acid . . . .
1 per cent. aqueous solution potassium sulphite (K_2SO_3) } Equal parts.

Differentiation is complete when the white matter is blue-black, the grey practically colourless.

8. Wash the sections in water. (Counter-staining in a carmine dye, or safranin, is optional after this stage.)
9. Dehydrate, clear, and mount (in dammar or balsam).

RELATIVE MERITS OF THE WEIGERT AND WEIGERT-PAL METHODS.—The Weigert (1891) method given here does not give so colourless a background of the grey matter as the Pal modification. Myelin sheaths are therefore rather sharper with the Pal technique. But the finer fibres—especially in the brain—are more easily decolorised by the latter. The Pal modification is more rapid.

Kultschitzky's Method.—The general principle would seem to be that of the Weigert method (see p. 255).

1. Fix in 4 to 10 per cent. formol for a few days.

2. Transfer thin (2 to 5 mm.) slices of the tissues to Weigert's rapid fixative (see p. 256).

Tissues are left in this for four to seven days.

3. Wash for a few hours in running water.

4. Dehydrate and embed in celloidin.

5. Stain in Kultschitzky's hæmatoxylin for twelve to twenty-four hours at room temperature.

The formula of this is :

Hæmatoxylin, saturated solution in absolute alcohol	10 c.c.
Glacial acetic acid	2 c.c.
Distilled water	100 c.c.

The hæmatoxylin solution must be ripe—*i.e.* kept in the sun and warmth for at least six weeks before being used. It should only be mixed with the acetic acid shortly before using it.

6. Differentiate in the following fluid :

Concentrated aqueous solution lithium carbonate .	100 c.c.
Potassium ferricyanide	10 c.c.

This process is very slow and should be controlled about once an hour with the staining microscope. For human cerebral cortex about four hours' treatment is usually necessary, for human spinal cord up to twelve hours. When the white matter is blue-black and the grey yellowish—

7. Wash the sections in running water.

8. Dehydrate, clear, and mount in dammar or balsam.

Myelin sheaths, black ; ground substance, yellow. Good for very fine fibres (*e.g.* in cerebral cortex).

Nageotte's Hæmalum Technique.—This has the advantage of rapidity, since frozen sections are used and the staining can be accelerated by heat.

1. Fix *either* in 4 per cent. formol, *or*, as advocated by Nageotte, in his special fluid, for two or three days.

40 per cent. formol . .	100 c.c.	} = Nageotte's fixative.
Aq. dest.	1000 c.c.	
Sodium sulphate (hydrated) .	10 to 70 grms.	

2. Wash the tissues in water.

3. Make frozen sections.

4. De-fat the sections by placing them for a few minutes in a dish of 90 per cent. alcohol slightly acidulated with acetic acid. (This is done because myelin, in frozen sections, is apt to be displaced during cutting.)

5. Stain in the following solution for half an hour at 50° C., or for twenty-four hours at room temperature :

Mayer's hæmalum (formula, p. 108) .	2 parts.
Absolute alcohol	1 part.

Filter before use.

6. Destain in a modified Weigert differentiator having the following composition :

Borax	5 grms.
Potassium ferricyanide . . .	2 grms.
Aq. dest.	100 grms.

A few minutes' treatment usually suffices. The right degree of differentiation has been attained when the grey matter is greyish yellow.

7. Rinse the sections in two changes of tap water, to the second of which a few drops of ammonia are added.

8. Transfer to distilled water.

9. Dehydrate, clear, and mount in dammar or balsam.

POSITIVE METHODS FOR DEGENERATED MYELIN

The methods of Weigert, Kultschitzky, and Nageotte do not stain degenerated myelin sheaths. Consequently, a degenerated tract appears as an unstained or nearly colourless area. Individual fibres, however, cannot be identified, if degenerate, with any certainty, since the tiny unstained space may easily be overlooked. Further, a negative staining reaction is less reliable than a positive one.

A fact which must again be emphasised is that the following methods are only applicable for a comparatively short time after degeneration has been initiated. They do not give positive pictures of long-staining degenerations, since the myelin of degenerated fibres is removed in course of time by phagocytes. Consequently the useful limits of these methods

are from ten days to two months (at the most) after the trauma (section or pressure) has been in action.

The Marchi Method.—The reaction on which this method is based is as follows: Normal myelin, since it is readily oxidised by potassium bichromate, does not stain with osmic acid after previous treatment with this chrome salt. But degenerated myelin contains oleic acid, which is not oxidised by the potassium bichromate, and hence readily stains with osmic acid.

1. Fix pieces of tissue, not more than 2 to 3 mm. thick, in the following fluid:

Müller's fluid	2 parts.
OR	
2·5 per cent. potassium bichromate .	
1 per cent. osmic acid . . .	1 part.

Spinal cord requires eight to fifteen days' treatment; brain and cerebellum, twenty-one to twenty-eight days. Change the solution every five or seven days, and place the tissues on cotton wool (or hang them by thread) to facilitate penetration.

NOTE.—Tissues may be transferred directly from formol to the potassium bichromate-osmic acid mixture, but formol fixation should not be prolonged beyond a few days.

2. Wash for twenty-four hours in running water.

3. Dehydrate rapidly in alcohol and embed as quickly as possible in celloidin. Or embed rapidly in paraffin.

4. Make sections of 7 to 12 μ.

5. Mount in dammar or balsam.

(Counter staining with safranin, neutral red, or van Gieson's stain is optional.)

Degenerated myelin, black; normal myelin, brownish. Nerve cells and neuroglia, unstained.

Artefacts and the Marchi Technique. — Roussy and Lhermitte have pointed out certain sources of error.

1. The tissues must be very carefully excised without squeezing, since degenerated myelin is easily displaced by pressure.

2. Over-fixation in formol or in Müller's solution may produce black granules (Marchi's pseudo-granulations).

3. The processes of dehydration and embedding must be run through as rapidly as possible, otherwise the stain is liable to be extracted.

4. Remember that the lipochrome granules of nerve cells may also be stained brown or black.

5. Fat cells of the connective-tissue stroma of nerve fibres are stained black.

Sudan III. (or Scharlach R) and Hæmatoxylin.—This method has the advantages of (*a*) far greater rapidity and (*b*) a more precise staining of degenerated myelin. It is specially useful as a complementary method for the study of the finer details of degeneration. The same remarks regarding the time limits of its application apply as to the Marchi technique.

1. Fix in 4 per cent. formol.

2. Make frozen sections after a preliminary soaking in water.

3. Place the sections for five minutes in 70 per cent. alcohol.

4. Stain in a saturated solution of Sudan III. in 70 per cent. alcohol for fifteen to thirty minutes. The solution should be preferably filtered immediately before using it. Or you can use the Scharlach R solution described on p. 169.

5. Rinse the sections in water.

6. Stain in Delafield (or Ehrlich's) hæmatoxylin.

7. Blue the hæmatoxylin in tap water, or in Scott's tap-water substitute.

8. Mould in glycerine jelly or glycerine.

NOTE.—(1) Avoid high-grade alcohol and clearing media, or the degenerated myelin will be dissolved.

(2) Some displacement of the degenerated myelin may occur during the manipulation of the frozen sections. Gelatine inclusion (see p. 87) should avoid this.

(3) The hæmatoxylin fades in course of time in glycerinated mounts.

Normal myelin, practically colourless ; degenerated myelin, orange (Sudan III.) or red (Scharlach R). Adipose tissue, deeply stained. Lipochrome pigment of nerve cells, red or yellow.

NEUROGLIAL METHODS

General Considerations.—The neuroglial system consists of neuroglial cells, their processes, and neuroglia fibres. The latter, although probably formed by the neuroglia cells, form a system which is largely distinct from the actual cells and their processes. Furthermore, certain staining methods have special affinity for neuroglia fibres rather than for the cell bodies. And the subject is rendered complex by the fact that there exist four different types of neuroglia, one of which (oligodendroglia) is extremely difficult to stain.

The sorting out of the various types of neuroglia has been very largely due to their study, consequent upon the introduction of appropriate methods, by the Spanish school. From a memoir by Penfield it appears clear that neuroglia may be grouped as follows :

I. Fibrous Neuroglia.—Almost exclusively found in the white matter. Neuroglia nuclei and cells comparatively rare (as compared with type II.), but fibres very highly developed. Whence the suitability of the methods especially intended to demonstrate neuroglia fibres (methods of Weigert, Lhermitte, Anglade and Morel) for studying fibrous neuroglia.

II. Protoplasmic Neuroglia.—Almost exclusively characteristic of the grey matter. Neuroglia cells and processes are abundant and highly developed, but the fibres are scanty (in contrast with type I.). The best methods for studying protoplasmic neuroglia are therefore those methods which stain the cell bodies and the cell processes (methods of the Spanish school).

III. Oligodendroglia (=Adendritic corpuscles or third element of Cajal).—Present in both grey and white matter but more frequent in the white. Their nuclei are stained by all standard methods, but the cell bodies and processes were only adequately studied and recognised as separate neuroglial elements by Del Rio Hortega in 1918. A special silver carbonate method is necessary to stain oligodendroglia.

IV. Microglia.—*Not* true neuroglia, since these elements are mesodermal in origin, and hence belong to the connective-tissue group of cells, in contrast with groups I., II., and III., which are ectodermal in derivation. More numerous in the grey than the white matter. The phagocytic cells known as " Gitterzellen " or " Scavenger cells," and previously thought to be neuroglia, are hence mesodermal elements and not true neuroglia.

A variant of the silver carbonate method of Rio Hortega, who discovered microglia, is used to demonstrate them.

From the above it will be clear that, here as elsewhere, methods have to be adopted to suit the type of cell or fibre it is especially desired to study. The fibrous neuroglial system of the white matter, in which fibres are predominant. is best studied by the methods of Weigert, Lhermitte, and Anglade and Morel. For protoplasmic neuroglia the Spanish methods are particularly useful. While in the case of oligodendroglia and microglia very special methods are requisite.

It must be remembered that the Golgi method for axis-cylinders and dendrites also often stains neuroglia cells and processes with great precision (see p. 251). But it does not give a specific staining reaction for neuroglia.

Freshness of the tissues is most important for work on neuroglia. The higher the temperature at which the body is kept after death, the sooner does autolysis of these very delicate elements set in. Generally speaking, the neuroglia cells and their processes suffer before the fibres.

Such material should be fixed not more than one to two hours after death in hot weather. Even at low temperatures autolysis will set in quite rapidly. The ordinary autopsy, in which tissues are fixed anything from twelve to forty-eight hours after death, is useless for the application of neuroglial methods.

Apart from their utility for general histological work, the methods for neuroglia cells and fibres are very important in neurological pathology. The identification of gliomata, and the study of sclerotic processes, of the central nervous system involve their use.

A. METHODS FOR NEUROGLIA FIBRES

1. Method of Lhermitte and Guccione

This technique is rapid and fairly certain. It was designed for work on human material, but I have had excellent results with monkey spinal cord which had been for over five years in formol. With guinea-pig brain and spinal cord I have been

less successful. It is a modification of the original Weigert method.

Explanation.—It seems probable that the principle underlying this method is the same as for the Weigert myelin sheath and the Weigert neuroglial methods. That is to say, as a result of the mordanting, some chrome compound is formed, especially in or around the neuroglia fibres, and this substance acts as a mordant for the stain. As to why the metallic compound should have a special affinity for neuroglia fibres is not known.

Manipulation of the sections must be done with a fine glass rod.

1. Fix in 4 or 5 per cent. formol for at least fifteen days.
2. Wash the tissues for a few hours in running water.
3. Make frozen sections, preferably thin.
4. Collect the sections in distilled water.
5. Place the sections for two hours in a saturated aqueous solution of mercuric chloride.
6. Transfer direct to the following fluid — a modified Flemming's solution—for twenty-four to forty-eight hours :

1 per cent. chromic acid . .	50 c.c.
1 per cent. osmic acid. . .	12 c.c.
2 per cent. acetic acid. . .	8 c.c.
Aq. dest.	30 c.c.

This process mordants the neuroglia fibres prior to staining.

7. Rinse the sections in a dish of distilled water.
8. Float one or more sections on to a slide ; then press them down with cigarette paper.
9. Flood the slide with the following stain :

Victoria blue (Grübler) .	1·5 grm.
Aq. dest. . . .	100 c.c.
Warm to dissolve the dye ; then filter.	

Gently warm the flooded slide above a flame till it steams. Then lay it aside to cool. Repeat this process at least twice.

10. Pour off the stain and flood the slide with Lugol's solution (formula, p. 117). Leave for one minute.
11. Blot carefully.
12. Pour aniline oil-xylol (equal parts) over the sections,

and control the differentiation with the microscope. When fully differentiated, the sections are nearly colourless to the naked eye, and are transparent against a black background. The neuroglial fibres are blue.

13. Eliminate the aniline by pouring pure xylol on to the slide. (This is important ; if any aniline remains in the tissues the sections will fade.)

14. Mount in dammar or balsam.

Neuroglia fibres, an intense blue ; nuclei of neuroglia and of connective-tissue cells, blue ; likewise red blood corpuscles and fibrin. Connective-tissue fibres and nerve cells, colourless. This method, while staining neuroglia fibres, does not stain their processes or cell bodies.

NOTES.—(1) If you are unable, even after repeated steaming of the stain, to stain the fibres, treat the sections, after stage 7, for twenty-four to forty-eight hours with a 5 per cent. solution of oxalic acid.

(2) *Modifications enabling collagen fibres to be stained.—Modification* 1. Stages 1 to 10 as above. Treat for a few seconds with a 1 per cent. aqueous solution of trypan red. Then differentiate in the standard way.

Modification 2. Stages 1 to 13 as above. Then stain the sections for a few (usually one or two) seconds with a 1 per cent. solution of eosin or erythrosin in absolute alcohol. Rinse immediately in xylol. The treatment with the counter-stain must be very rapid, since alcohol destains the neuroglia fibres.

With both of these variants, collagen fibres and blood vessels are pink ; cell bodies of neuroglia, more or less stained ; neuroglia fibres, blue.

2. Method of Anglade and Morel

This technique, of which the preceding is a modification, allows of paraffin sections being made. Da Fano reports good results with lower animals, though the method was primarily intended for human nervous system.

1. Fix for twenty-four to forty-eight hours in 3 parts of Solution A to 1 of B :

1 per cent. chromic acid	25 c.c.	= Solution A (Fol's fixative).
1 per cent. osmic acid .	2 c.c.	
2 per cent. acetic acid .	5 c.c.	
Aq. dest. . . .	68 c.c.	

Saturated aqueous solution of mercuric chloride = Solution B.

2. Wash overnight in running water.

3. Dehydrate, clear, and embed in paraffin.

4. Stain sections on the slide with Victoria blue as for Lhermitte's method (see p. 265).

5. Treat with Gram's iodine or Lugol's solution for one minute.

6. Blot carefully.

7. Differentiate in aniline oil, 2 parts ; xylol, 1 part ; as by Lhermitte's method.

8. Wash in xylol.

9. Mount in dammar or balsam.

Neuroglia fibres, blue ; nerve cells, greenish.

3. Weigert's Method for Neuroglia Fibres

This was the classical method, of which the above are variants. Since it has the disadvantages of being very long, capricious, and only applicable to the human subject, it is not described. It may be advantageously replaced by the methods already described, also by the silver carbonate method of Rio Hortega, Modification II., p. 268.

B. METHODS FOR NEUROGLIA CELLS AND THEIR PROCESSES

The methods of Cajal, Rio Hortega, and Achucarro are especially recommended. They are applicable to human material and also give good results with other species—particularly mammalian. In successful preparations the precision of the staining is extraordinary.

All these methods are primarily of the photographic type, for an explanation of which, see p. 142. Frozen sections are the rule.

RIO HORTEGA'S SILVER CARBONATE METHOD

Modification 1.—*For Protoplasmic Neuroglia.*

1. Fix pieces of very fresh tissue in Cajal's ammonium bromide-formalin mixture for fifteen to thirty days at room temperature.

40 per cent. neutral formalin	15 c.c.
Aq. dest.	85 c.c.
Ammonium bromide	1·5 to 2 grms.

2. Cut frozen sections of 15 to 20 μ.

3. Wash in two changes of distilled water.

4. Place the sections in a capsule of the silver carbonate solution which is heated above a flame, or in the embedding thermostat, to 45° or 50° C. Staining is complete when the sections have turned a greyish yellow—a matter of a few minutes.

Preparation of the Silver Carbonate.—To 10 c.c. of a 10 per cent. aqueous solution of silver nitrate add an equal volume (or slightly more) of a saturated solution of pure lithium carbonate. A precipitate is formed. Collect this on a filter paper; wash it by passing about 50 c.c. of distilled water through the filter paper. Then transfer the precipitate to 15 or 20 c.c. of distilled water. Cautiously add ammonia till the precipitate is just dissolved. Then make up to 50 c.c. with distilled water.

The reactions involved are (Gorriz):

(1) $2AgNO_3 + Li_2CO_3 = Ag_2CO_3 + 2LiNO_3$ (removed by washing).

(2) On dissolving the precipitate:

$Ag_2CO_3 + 4NH_3 = [Ag(NH_3)_2]_2CO_3$ (ammonium silver carbonate).

5. Rinse the sections for a few moments in distilled water. Rock the dish. (*N.B.*—If you wash for too long, the impregnation will be weak.)

6. Reduce in 20 per cent. formol for half a minute.

7. Wash thoroughly in distilled water.

(*a*) Toning (optional). Place the sections in a warm (*circa* 45° C.) solution of 0·2 per cent. gold chloride till they turn violet.

(*b*) Fixation (optional). Transfer for one minute to a 5 per cent. solution of sodium thiosulphate ("Hypo"). Then wash in water.

8. Dehydrate, clear, and mount in dammar or balsam.

Note.—If you fix in 10 per cent. formol (*i.e.* omit treatment with the ammonium bromide), and then proceed as in Modification I., the processes of nerve cells are stained instead of the neuroglia.

Modification II.—*For Fibrous Neuroglia.*

Fix for *twenty to forty days* in the ammonium bromide-formalin mixture (p. 267). Then proceed as by Modification I.

Modification III.—*For Oligodendroglia* [=Adendritic cells or third element of Cajal (partly); "Mesoglia" of Robertson (obsolete)].

1. Fix for twelve to forty-eight hours in Cajal's ammonium bromide-formalin solution (formula, p. 267).

2. Bromuration. Warm the pieces of tissue for ten minutes at 45° to 50° C. in a fresh bath of mixture.

3. Wash in water for a few hours. This eliminates the formol and hence facilitates freezing.

4. Make frozen sections 15 to 20 μ in thickness.

5. Wash the sections in two changes of distilled water. A few drops of ammonia may advantageously be added to the first bath.

6. Impregnate for one to five minutes in the following solution:

10 per cent. silver nitrate solution . . .	5 c.c.
5 per cent. pure sodium carbonate solution .	20 c.c.
Add ammonia till the precipitate is dissolved.	
Add distilled water to make up to . . .	45 c.c.
Filter and keep in a dark glass bottle.	

7. Rinse the sections for fifteen seconds in distilled water.

8. Reduce in 1 per cent. formol solution.

9. Rinse in distilled water.

10. Tone in an 0·2 per cent. solution of (yellow) gold chloride, till the sections are grey (usually ten to fifteen minutes).

11. Fix the impregnation in 5 per cent. sodium thiosulphate ("Hypo") for one minute.

12. Wash in water.

13. Dehydrate in 95 per cent. alcohol.

14. Clear in carbol-xylol-creosote:

Phenol cryst.	10 grms.
Xylol	80 c.c.
Pure creosote	10 c.c.

15. Mount in dammar or balsam.

Note.—Penfield recommends, especially for the central nervous system of rabbits, two to twelve hours' treatment in the ammonium bromide-formol, directly followed by thirty-six to forty-eight hours'

post-fixation in 95 per cent. alcohol, bromuration being omitted. Impregnation is prolonged for from fifteen minutes to two hours (until the sections begin to turn brown). Other stages as above.

MODIFICATION IV.—*For Microglia* [=Rio Hortegazellen; third element or adendritic cells (partly) of Cajal; Stäbchenzellen and Gitterzellen of Pathology].

1. Pieces of tissue 2 to 3 mm. thick are fixed for one to two days at 35° C., *or* two to four days at room temperature in:

Ammonium bromide . .	10 grms.
40 per cent. formol . . .	70 c.c.
Distilled water . . .	430 c.c.

2. Warm the pieces of tissue for ten minutes at 50° to 55° C. in a fresh volume of the fixative.

3. Make frozen sections 25 to 30 μ thick.

4. Rapidly rinse the sections in two changes of distilled water, to the first of which 2 drops of ammonia are added to 50 c.c. of distilled water.

5. Treat for ten minutes at 15° to 20° C. in the following silver carbonate solution:

10 per cent. silver nitrate solution . . .	5 c.c.
5 per cent. pure sodium carbonate solution . .	20 c.c.

Add ammonia drop by drop till the yellow precipitate is just dissolved. Then add 15 c.c of distilled water

6. Reduce in a 10 per cent. solution of acid-free formalin. Move the sections during this process. A simple expedient is to place them in a large Petri dish and blow gently on the fluid.

7. Wash in distilled water.

8. Tone } as for Modification III.
9. Fix }

10. Dehydrate in 95 per cent. alcohol.

11. Clear in carbol-xylol-creosote (formula, p. 269).

12. Mount in dammar or balsam.

Microglia, deeply stained; protoplasmic and fibrous neuroglia, likewise oligodendroglia and glia fibres, faintly stained.

CAJAL'S SUBLIMATE-GOLD METHOD

An excellent technique, giving especially good results with the human subject. Also applicable to the dog and cat, less well to the rabbit. Protoplasmic and fibrous neuroglia are stained, but neither microglia nor oligodendroglia.

1. Fix fresh pieces of tissue for two to six days in the ammonium bromide-formol fixative (formula, p. 267).
2. Make frozen sections of 20 to 30 μ in thickness.
3. Rinse the sections in two or three changes of distilled water.
4. Place the sections for four to eight hours at 18° to 20° C. in the dark in the following solution :

1 per cent aqueous solution of gold chloride (*brown variety of Merck*) . . .	60 c.c.
Pure crystallised mercuric chloride ($HgCl_2$)	0·5 grm.
Pure distilled water	10 c.c.

The solution must be used within a few hours of mixing the gold chloride and mercuric chloride. Impregnation is best done in capsules of about 6 cm. diameter and about 1 cm. depth of fluid. Do not attempt to impregnate more than four to six sections for every 15 c.c. of fluid. The sections are preferably applied to the bottom of the dish with a clean camel's-hair brush.

See also NOTES on next page.

When the sections are deep purple :

5. Rinse in distilled water.
6. Fix for five to ten minutes in the following bath :

Sodium thiosulphate (= " Hypo ") . . .	5 grms.
Distilled water	70 c.c.
Absolute alcohol	30 c.c.
Saturated aqueous solution of sodium bisulphite .	5 c.c.

7. Wash in 50 per cent. alcohol (diluted with distilled water).
8. Float the section or sections on to a clean slide.

Blot carefully with cigarette or good quality filter paper.

9. Absolute alcohol.
10. Carbol-xylol or origanum oil.
11. Dammar or balsam.

Neuroglia cells, purple red; nerve cells, pinkish; nerve fibres, nearly or quite unstained.

NOTES.—(1) Purity of the gold chloride and mercuric chloride is essential for successful results. *The brown variety of gold chloride manufactured by Merck must be used.* This solution keeps for months *in the dark.*

N.B.[1]—Various salts of gold are sold under the name of "gold chloride." Merck's aurum chloratum fuscum (brown gold chloride) is the hydrated gold chloride $AuCl_3.2H_2O$, containing 58 per cent. of gold, and it alone is suitable for this method.

Merck's aurum chloratum flavum (yellow gold chloride) is hydrated chlorauric acid, $HAuCl_4.3H_2O$, and contains 50 per cent. of gold.

The so-called "Pure" English gold chloride is the hydrated sodium chloraurate, $NaAuCl_4.2H_2O$, and contains 49·6 per cent. of gold.

"Sodium gold chloride" is a mixture of sodium chloraurate and sodium chloride in equal parts.

(2) The temperature at which impregnation is effected is important. 15° to 18° C. is the optimum. At 12° C. or less the results are poor and inconstant. At 24° C. impregnation is rapid (one to two hours), but coarse.

(3) The distilled water must be glass-distilled.

PERIPHERAL NERVOUS SYSTEM

The structures studied under this heading fall into two groups:

Nerve fibres (medullated and non-medullated);
Nerve endings.

For nerve fibres the methods employed for their study in the central nervous system are applicable. In the case of nerve endings special, and difficult, methods are required.

MYELINATED NERVE FIBRES

Osmic Acid Method.—This is one of the best and simplest methods for observations on both normal and degenerated myelinated fibres. Proceed thus:

VARIANT I.—*For Teased Preparations.*

1. Carefully excise the nerve and place it upon a piece of

[1] [Note by F. Haynes.]

filter paper, which is then floated, nerve downwards, on to the surface of a 1 per cent. solution of osmic acid in a small capsule. Leave therein for twenty-four to seventy-two hours.

2. Wash in water for several hours.

3. Preserve in dilute glycerine (distilled water and glycerine, equal parts).

4. To make microscopic preparations, carefully dissociate the nerve with fine needles on a slide.

5. Mount in glycerine jelly or glycerine.

Myelin, black to dark grey. Degenerative phenomena, from the tenth day to about six weeks after the onset of degeneration, are well shown.

VARIANT II.—*For Sections.*

Stages 1 and 2 as above.

Dehydrate in progressive concentrations of alcohol. The baths of 50 per cent. and 70 per cent. alcohol should be prolonged (*i.e.* twelve to twenty-four hours) while treatment with 90 per cent. and absolute alcohol should be cut down to the minimum time consistent with dehydration.

Clear in chloroform } rapidly.
Embed in paraffin }

Sections on the slide are mounted in thick chloroform balsam. Counter-staining on the slide in safranin is advantageous.

NOTES.—(1) You prolong the treatment with low-grade alcohol because osmicated myelin is thereby rendered more resistant to the solvent action of high-grade alcohol and the clearing medium.

(2) Provided thick balsam be employed, and that it be dried rapidly on the hot plate, such preparations are fairly permanent.

(3) You can also mount, after removal of the paraffin and passage through the alcohols down to water, in dilute glycerine or in glycerine jelly.

The Weigert and Weigert-Pal Methods are used for segments of nerve as for medullated fibres of the central nervous system (*q.v.*, p. 255).

The Marchi Technique is similarly employed (see p. 261).

The Bielschowsky Method is applicable to the peripheral nervous system. But to get good results, the variant outlined

below is necessary in order to weaken the staining of connective tissue.

The general indications on p. 248 regarding the preparation of the ammoniacal silver oxide solution, etc., are to be followed.

1. Fix pieces of nerve, etc., in 10 per cent. acid-free formol for twenty-four hours.
2. Wash in water.
3. Make frozen sections of 5 to 10 μ in thickness.
4. Collect the sections in distilled water.
5. Impregnate in 2 to 3 per cent. silver nitrate for twenty-four hours.
6. Rinse the sections for two or three seconds in distilled water.
7. Treat with the ammoniacal silver oxide solution for ten to twenty minutes. The sections will turn brownish yellow.
8. Place them in the following solution till pale yellow (usually two to five minutes) :

Glacial acetic acid	1 to 3 drops.
Distilled water	20 c.c.

9. Reduce in 10 per cent. neutral formol for five to thirty minutes.
10. Rinse the sections rapidly in distilled water.
11. Tone in :

1 per cent. gold chloride solution . .	5 drops.
Distilled water	10 c.c.

12. Fix in 5 per cent. sodium thiosulphate (" Hypo ") for thirty seconds.
13. Wash well in distilled water.

Dehydrate, clear, and mount in dammar or balsam.

Axis cylinders, black ; connective tissue, a brownish violet. Nerve endings may also be stained by this modification, but the results with these are capricious.

NON-MYELINATED FIBRES

The methods of Bielschowsky and Cajal stain both myelinated and non-myelinated fibres. The following is fairly specific for the latter :

Ranson's Method.—A modification of the Cajal technique for neurofibrils. Good results have been reported with man, the dog and the cat.

1. Fix segments of nerve for forty-eight hours in—

Absolute alcohol	99 c.c.
Concentrated ammonia	1 c.c.

2. Rinse the tissues in distilled water.
3. Place in pyridine for twenty-four hours.
4. Place in many changes of distilled water for twenty-four hours.
5. Place in 2 per cent. silver nitrate solution for three days in the dark.
6. Rinse in distilled water.
7. Reduce for several hours in—

Pyrogallol	4 grms.
5 per cent. formol in distilled water	100 c.c.

8. Wash in water.

Dehydrate, clear, embed in paraffin, and cut sections.

Non-myelinated fibres, black; myelinated axons, yellow; endoneurium, pale yellow.

NERVE ENDINGS

The staining or impregnation of these structures is difficult. The same technique, applied on different occasions to the same tissue, often yields quite different results. Observations on end organs therefore entail the making of a large number of preparations preferably by more than one method.

A selection of standard methods is given below, but it will often be found that the duration and, possibly, the strengths, of the various baths will need alteration. Work on nerve endings is largely done by the trial-and-error method, and a given technique should be repeatedly used with slight variations until the best percentage of successful preparations is attained. Even in the hands of experts this percentage is sometimes surprisingly small.

The methods for nerve endings groups themselves into—

I. Metallic impregnation methods.
II. Intravitam staining methods.

I. Metallic Impregnation Methods

Ranvier-Loewit Gold Chloride Method.—An old but excellent method for nerve endings in striated muscle, in the cornea, and in skin.

1. Fix small pieces of tissue for five to fifteen minutes in lemon juice filtered through flannel. The juice must be fresh and taken from ripe lemons.
2. Rinse rapidly in distilled water.
3. Place for twenty to sixty minutes in a 1 per cent. solution of gold chloride.
4. Rinse rapidly in distilled water.
5. Reduce for twenty-four hours in :

Formic acid	1 part.
Distilled water . . .	2 parts.

6. Wash in distilled water.
7. Mount either in glycerine jelly (or dilute glycerine) *or* in balsam. In the latter case some shrinkage of the muscle fibres will occur during dehydration.

Nerve fibres and nerve endings, violet to black ; muscle fibres (usually), reddish.

Modifications.—Sometimes better results are obtained, especially in the case of motor end-plates in muscle—(*a*) by placing the muscle directly in the gold chloride ; (*b*) by fixing in 25 per cent. formic acid for five to fifteen minutes and then treating with gold chloride.

The Ranvier methods are best for thin sheets of muscle or dissociated preparations.

For sectional work the Cajal neurofibril method, particularly Variants Nos. II. and V., is indicated. See p. 247.

The Bielschowsky method for peripheral nerves (see pp. 273–4) can also be applied to nerve endings.

For nerve endings and nerve cells in teeth, see *Mummery's method*, p. 297.

II. Intravitam Methods

These methods involve the action of methylene-blue on perfectly fresh tissues. The general remarks on intravitam staining (p. 191) are applicable here.

VARIANT I.—For the intravitam examination of thin sheets of dissociated preparations of muscle, etc.

1. Place the tissues either on a slide or in a watch-glass in a 0·25 to 0·03 per cent. solution of pure methylene-blue in isotonic saline. A solution of 0·05 per cent. is often satisfactory, and may be used in the initial attempt. Cover the slide with a watch-glass bearing a piece of moistened filter paper; if the tissues are in a watch-glass or capsule, see that they are only *just* covered by the methylene-blue. Access of air is essential for staining to occur. Leave for fifteen to thirty minutes.

2. Examine in normal saline solution with the microscope. If nerve endings are *not* stained, repeat the staining process, or modify the concentration of the dye; *if stained*—

3. Add a cover-slip and rapidly examine intravitam.

Nerve fibres (myelinated and non-myelinated) and their arborisations in end organs, are blue; the other tissue constituents should only be faintly stained. Muscle fibres, in successful preparations, are nearly colourless.

NOTES.—(1) Intravitam observations on unfixed specimens must be made rapidly, as the dye fades soon after the preparation has been covered. But the stain can be usually resuscitated by exposing the tissue to the air for a few minutes by removal of the cover-slip.

(2) In the case of muscle the tissues must be very delicately manipulated before and during intravitam staining. Damaged muscle fibres—as evidenced by cut or compressed fibres in dissociated specimens—stain a deep blue colour which completely masks the staining of nerves and nerve endings.

VARIANT II.—*Fixation of the Stain.*—The following method is perhaps one of the best:

Stages 1 and 2 as for Variant I.

3. Transfer the tissue to a saturated aqueous solution of ammonium picrate. (*N.B.*—Do not attempt to grind up the crystals of this substance before dissolving it, as it is highly explosive.) Leave therein for one-half to one hour.

4. Place direct in:

Ammonium molybdate . .	1 grm.
Distilled water . . .	20 c.c.
Hydrochloric acid . . .	1 drop.

Leave therein for one or more hours.

5. Wash in distilled water.

6. Dehydrate as rapidly as possible in ascending grades of alcohol. Less of the stain is extracted by dehydrating at a fairly low temperature—*circa* 10° C. In any case it is safer not to prolong dehydration beyond thirty to fifty minutes.

7. Clear in xylol.

8. Mount in dammar or balsam.

NOTE.—Fixation of the methylene-blue is capricious. Sometimes better results are obtained by mounting, after stage 5, in the following medium :

Saturated aqueous solution of ammonium picrate Pure glycerine	Equal parts.

VARIANT III.—*For Sections.*—Proceed as for Variant II. up to stage 7.

Impregnate with paraffin for thirty minutes to one hour.

Make sections.

Counter-staining with neutral red or safranin is optional. Treatment with alcohol during manipulation of the sections must be as short as possible.

For further details as to methods for nerve endings, the excellent account in Romeis, *Taschenbuch der Mikroskopischen Technik*, may be consulted.

NUCLEOLI

Standard cytological stains may be used. Iron-hæmatoxylin and eosin-methylene-blue are particularly useful. For determining the oxy- or basiphility of these bodies, Unna's pyronin-methyl-green (p. 145) is of service.

Nucleolini.—These bodies may be seen after very careful differentiation of sections stained in Heidenhain's iron-hæmatoxylin, or in eosin-methylene-blue, -toluidin-blue, -thionin. A water-immersion objective is extremely useful to control the degree of extraction. In the case of the nucleolini of plasmosomes, very prolonged differentiation is needed for Heidenhain's iron-hæmatoxylin.

The Cajal uranium nitrate-formol method for the Golgi

bodies very frequently also impregnates, with extraordinary clearness, the nucleolini. It may be specially modified so as to give a nearly specific stain for these bodies in the frog (Carleton).

OVARY

(See Sex Glands, p. 284)

PANCREAS AND SALIVARY GLANDS

Suitable fixatives for micro-anatomical work are mercuric chloride-formol, Zenker's fluid, and 4 per cent. formol in 0·9 per cent. sodium chloride. For cytological work the fluids of Flemming and Regaud are very good. For the study of the intracellular granules acetic acid as a constituent of the fixative must at all costs be avoided, since it destroys zymogen and other granules. For mucin in salivary glands, see p. 176.

Standard histological stains are applicable. The islet cells of the pancreas are particularly well shown after fixation in mercuric chloride or mercuric chloride-acetic acid followed by pyronin-methyl-green, which stains them red. Mallory's connective-tissue stain also demonstrates the islet tissue very clearly, staining it red.

The mitochondria may be studied in formol, or, better, in Regaud-fixed specimens stained in Heidenhain's iron-hæmatoxylin. The Regaud-Kull technique (p. 140) is also useful.

To demonstrate the Golgi elements, Da Fano's modification of the original Cajal method (p. 142) is excellent.

To distinguish between the "A" and "B" cells of the pancreatic islet tissue, Lane has devised the following technique:

1. Fix small fragments of the gland for three to four hours in the following fluid:

Potassium bichromate	2·5 grms.
Mercuric chloride	5 grms.
Distilled water	100 c.c.

(The sodium sulphate of the original formula may be omitted.)

2. Dehydrate, clear, and embed in paraffin.
3. Cut sections 3 to 5 μ in thickness.

4. Stain for twenty-four hours in Bensley's neutral gentian,[1] which is prepared as follows :

Dissolve 1 gramme of gentian violet in 25 c.c. of distilled water.
Dissolve 1 gramme of orange G in 25 c.c. of distilled water.
Mix and shake ; a precipitate is formed which is separated by filtration, washed once with distilled water, and dried. Finally it is dissolved in 25 c.c. of absolute alcohol. This is a stock solution. For staining add sufficient of it to 20 per cent. alcohol to give a deep violet liquid.

5. Blot the sections.
6. Dehydrate and partly differentiate by a few seconds' treatment with absolute alcohol.
7. Blot rapidly.
8. Flood the slide with oil of cloves and control differentiation with the staining microscope. When the *acinous cells* show purple zymogen granules and their ground cytoplasm is brownish yellow :
9. Wash the slide in xylol.
10. Mount in dammar or balsam.

" A " Cells.—Cytoplasm, barely stained ; nuclei, pale purple.

" B " Cells.—Cytoplasmic granules, a brilliant purple ; nuclei, purplish.

Acinous Cells.—Zymogen granules, purple ; ground cytoplasm, yellow.

The above staining reaction may be reversed, and the granules of the " A " cells stained violet while the " B " cells are relatively unstained, by fixing for two hours in the following solution :

Saturated solution of mercuric chloride in 95 per cent. alcohol, and 3·5 per cent. aqueous solution of potassium bichromate, equal parts.

Then proceed with stages 2 to 10 as above, using iodine and " Hypo " to remove the mercury deposit (see pp. 124 and 125).

Note.—Fixation in 70 per cent. alcohol for twenty-four hours may be substituted for the above mixture. Staining results are similar.

[1] For an explanation of neutral dyes, see p. 94.

PIGMENT GRANULES

See sections on pigment in "Histo-Chemical Methods," p. 178; also "Pigmentation (Pathological)," p. 315.

PITUITARY BODY

Care must be taken not to injure this organ when removing it.

Suitable fixatives, for micro-anatomical purposes, are 5 per cent. formol in 0·9 per cent. sodium chloride, mercuric chloride-formol, and Zenker's fluid. Paraffin sections, usually in the sagittal plane, are to be preferred to celloidin. Standard histological stains can be used.

Anterior Lobe.—The oxyphil and basophil cells are well shown by eosin-methylene-blue or Mallory's connective-tissue (fuchsin-orange G-aniline blue) stains. The mitochondria are demonstrated by Heidenhain's iron-hæmatoxylin following fixation in Regaud's fluid, or by the Regaud-Kull technique. The Golgi elements are impregnated by Cajal's uranium nitrate-formol process, or by Da Fano's modification of it.

Posterior Lobe.—The neuroglial network of fibres is stained by one of the special methods for these fibres. See section on these methods, p. 264.

PLASMA CELLS

The round nucleus, with its chromatin arranged in radial masses, and the voluminous, oval cytoplasm, usually enables these cells to be easily recognised by any method. Further, the cytoplasm is non-granular and basophil.

Unna has devised several methods which demonstrate plasma cells with great clarity. The following technique is very good:

1. Fix in absolute alcohol. Fair results are given after fixation in mercuric chloride, mercuric chloride-formol, and formol.
2. Paraffin or celloidin sections.
3. Stain for twenty to forty minutes in Unna's pyronin-methyl-green (formula, p. 112).

4. Rinse the sections in water.
5. Blot them carefully.
6. Dehydrate very rapidly in absolute alcohol.
7. Xylol.
8. Dammar or balsam.

Cytoplasm of plasma cells, red ; their nuclei, bluish green. Lymphocytes stain in the same way but are easily distinguishable morphologically.

SEROUS MEMBRANES AND ENDOTHELIA

Serous Membranes are best studied not in sections, but in small spread-out strips. In this way the cells and their outlines (if impregnated with silver nitrate) may be studied even with the highest powers.

To make such preparations, stretch a piece of the membrane to be examined over a stout segment of wide-bore glass tubing. Then tie it on. This is often best performed *in situ* ; the segment of tube or ring is held in one hand, the membrane is stretched over it with the other, while an assistant passes and quickly ties a loop of thread around the tube. The latter is then cut away from the remainder of the membrane. The stretched-out membrane may then be fixed and stained by any standard method. Just before mounting, the tube is inverted on a slide and the membrane freed from it by cutting it away.

To study the cell outlines in such tissues, impregnation with silver is necessary. Mann's method often gives excellent results.

1. *Either* stretch the tissue over a segment of tube *or* spread it out, and partially allow to dry to cause adhesion, on a slide.

2. Treat for about two minutes with a 5 per cent. solution of silver nitrate, wash in a stream of distilled water, and treat again with the silver solution.

3. Place the tissue—still in the silver nitrate—in the sun until brown.

4. Wash thoroughly in distilled water to remove any free silver salt. If this step be omitted, progressive deposition

of a black precipitate of silver may occur after the specimen has been mounted.

5. Counter-stain the nuceli in an 0·5 per cent. aqueous solution of toluidin-blue for about a minute (optional).

6. Dehydrate, clear, and mount.

NOTES.—(1) The times required for impregnation, the strength of the silver solution, and the duration of exposure to light, are very variable.

(2) Reduction is hastened by placing the tissues on a piece of white paper and not on the (usually) dark surface of the laboratory bench.

(3) Failing sunlight, I have found a 50 c.p. Mazda electric bulb an efficient—if not a better—substitute. Or you may proceed direct to balsam, after impregnation and washing rapidly in distilled water, and then expose to the light. This method I have found particularly efficacious with sections.

(4) The nature of the silver deposit in and around the cement substance of cells is unknown. According to Mann, it is probably a silver-proteid combination with the chlorides and carbonates which are present in higher concentration in the intercellular spaces than elsewhere. It apparently is not metallic silver.

Endothelium.—Pieces of this may be stripped from the larger blood vessels and treated as above. To study the cell outlines *in situ* Mann's method may be used.

1. Wash out the blood vessels of a just-killed animal with an 0·25 to 0·125 per cent. solution of potassium nitrate (K_2NO_3). Any large vessel may be selected (*e.g.* the aorta); a glass cannula is tied in. With the aid of an indiarubber tube and a fair-sized funnel the blood is washed out from the vessels. The pressure required varies considerably, but 1 to $1\frac{1}{2}$ metres (of water) is usually sufficient.[1]

2. Follow up with an injection of an 0·5 per cent. solution of silver nitrate to every 100 c.c. of which 1 drop of nitric acid has been added. Prolong this injection for three to five minutes.

3. Inject a warm 5 per cent. solution of gelatine in distilled water, in order to prevent the blood vessels from collapsing. When the animal is cold:

4. Excise the tissues you wish to study and fix them for twenty-four hours in 95 to 96 per cent. alcohol.

[1] The detailed procedure for injection is given on p. 185 *et seq.*

5. Make thick frozen sections after soaking in gum.

6. Place the sections in 70 per cent. alcohol and expose to sunlight to reduce the silver.

7. Stain the nuclei, if desired, in any basic aniline dye (*e.g.* in 0·25 per cent. toluidin-blue).

8. Dehydrate, clear, and mount in dammar or balsam.

SEX GLANDS

Preservation.—For any observations of a cytological nature, the ovary or testis must be fixed immediately after death. It must also be borne in mind that degenerative changes occur in the testis in the course of many diseases. Absolute freshness of the material is a *sine qua non* for counting chromosomes. For micro-anatomical purposes the gonads as procured from the post-mortem room are useful enough. But the germinal epithelium of the ovary is usually absent from autopsy specimens owing to the desquamation after death, while in the case of ovaries excised at operation it is often stripped away during the removal of the organ.

For a more detailed account of suitable methods (especially cytological) for the germ cells, consult the excellent article by Gatenby in the *Microtomist's Vade Mecum* (1921).

Fixation.—Small ovaries can be fixed in bulk ; for larger ones the organ should be transversely bisected. The testis, on account of the low permeability of the tunica vaginalis, should be sliced up with a very sharp knife prior to fixation.

Ovary.—Suitable fixatives for general work are :

Mercuric chloride-formol.
Zenker's fluid.
4 per cent. formol in 0·9 per cent. sodium chloride.
Bouin's fluid.

For cytological observations, Regaud's formol-bichromate, Gatenby's Flemming without acetic acid, and Champy's fluid are useful for fixing mitochondria. The subsequent staining should be Heidenhain's iron-hæmatoxylin or the Kull method.

The Golgi elements are shown by the Cajal uranium nitrate-formol method or Da Fano's modification of it.

Embedding in paraffin is preferable to celloidin, since paraffin allows thin sections to be made. Human ovaries —especially after the menopause, when the fibrous tissue of the stroma hypertrophies—must be embedded in hard paraffin (m.p. of 55° C.) and the duration of the embedding process shortened so far as is possible. Avoid xylol as a clearing medium, and use cedar oil or benzol instead.

General histological stains can be used. One of the best methods to demonstrate the microscopic anatomy of ovary is Mallory's fuchsin-aniline-blue-orange G stain after Zenker or mercuric chloride-formol fixation.

Isolated Ova may be obtained from the Graafian follicles as follows :

A fresh ovary is excised : mature follicles, if present, will be seen as thin-walled swollen areas on the surface of the ovary. Neither a pregnant nor an immature subject is suitable. The follicular wall is punctured with any sharp instrument and the exuding fluid taken up on a slide. Or the ovum may be obtained by introducing a capillary pipette into the follicular cavity. Sometimes slight pressure has to be exerted around the follicle, in which case the ovary should be immersed in a shallow capsule of warm Ringer-Locke solution.

The ova may be examined as fresh preparations or else fixed in Zenker's fluid, Regaud's formol-bichromate, or such other fixative as may be indicated. Dehydration is carefully graded from 30 per cent. alcohol upwards and the ova are orientated on a piece of liver, beneath the dissecting microscope, by Hill's method (p. 222). They are embedded in paraffin and stained. Whole stained preparations of ova are not successful.

Testis.—Suitable fixatives for general work are :

Zenker's fluid.
Zenker's formol.
Mercuric chloride-formol.
Bouin's fluid.

Formol fixation is generally inferior in its results as compared with the four above fluids.

Chromosomes are best fixed by Bouin's fluid, or by the fluids of Flemming (very thin slices of tissue) or Carnoy.

Absolute freshness of the tissue is essential for reliable counts on chromosomes. For these and most cytological observations, Heidenhain's iron-hæmatoxylin is quite the best stain.

For demonstrating mitochondria, fixation in Regaud's formol-bichromate mixture gives better results for mammalian testis than the fixatives of Champy or Gatenby. Heidenhain's iron-hæmatoxylin or Kull's method are suitable stains.

The Golgi elements can be impregnated by the Cajal uranium nitrate-formol technique or by Da Fano's modification of it.

SKIN (INCLUDING HAIR AND NAILS)

The special methods for skin largely arise from the need (*a*) for very thin sections (2 to 4 μ), and (*b*) for the differential staining of the various elements (cells, fibres, intercellular bridges, etc.) which are comprised in that very complex tissue, the epidermis. The necessity for thin sections is especially important in fine histological work, for here, unless the minimum of thickness is attained, it is impossible to make out the relations between epidermal cells and fibres owing to the super-imposition of these elements.

Suitable Fixatives

Zenker's fluid.
Mercuric chloride-formol.
5 per cent. formol in 0·9 per cent. sodium chloride.
Bouin's fluid.
Absolute alcohol (chiefly for histo-chemical researches).

Skin is not very sensitive to post-mortem changes, and quite satisfactory micro-anatomical pictures are furnished by material from the post-mortem room. But for cytological purposes specimens must be preserved soon after death. Material obtained at operation is often useful, as when a pathological (*e.g.* epitheliomatous) area of skin is excised, a margin of normal tissue is taken with it.

Pieces of skin, other than thin shreds, should be pinned out flat, but without undue distension, by hedgehog quills on a paraffined cork.

Dehydration, Clearing, and Embedding of Skin

This is notoriously difficult if very thin sections are to be obtained. The two following methods, recommended by Shapiro, give excellent results :

(*a*)	5 per cent. formol	24 hours.
	Absolute alcohol	24 „
	Acetone	1 hour.
	Paraffin (50° C.)	6 hours.
(*b*)	Zenker (or mercuric chloride-formol)	24 hours.
	Alcohol (75 per cent., 95 per cent., absolute)	8 hours in each.
	Cedarwood oil	24 hours.
	Ligroin	12 „
	Ligroin-paraffin (on top of the embedding bath (*i.e.* at 20° to 25° C.)	12 „
	Paraffin (50° C.)	6 „

Xylol and chloroform should not be used as clearing agents, since they make skin extremely brittle. Too short a treatment with paraffin must also be avoided ; owing to the closeness of its constituent cells, the permeation of a piece of skin by the paraffin is considerably slower than in the case of tissues of a looser texture.

To obtain satisfactory sections of large areas of skin—especially if there be much keratinisation—celloidin embedding is to be preferred. Difficult material may often be cut quite thin on a sliding microtome after collodionisation of the block (see p. 83).

For rapid (*e.g.* diagnostic) work, the freezing microtome is useful.

Staining Methods

For micro-anatomical purposes standard staining methods may be used. Very valuable results are given by Heidenhain's iron-hæmatoxylin.

Special Methods

Epithelial Fibres (=Ereidesms of Shapiro=Intracellular Fibres).

The following methods are useful for demonstrating these structures :

A. *Unna's Water Blue-Orcein-Safranin Method*

Fix the skin in 5 per cent. formol or absolute alcohol.

Make thin paraffin or celloidin sections.

1. Stain for ten minutes in a large watch-glass containing 10 parts of solution A, 3 of B, and 3 of C.[1]

SOLUTION A

Water blue	1 grm.
Orcein (of Grübler)	1 grm.
Acetic acid (glacial)	5 c.c.
Glycerine	20 c.c.
Absolute alcohol	50 c.c.
Distilled water	100 c.c.

SOLUTION B

Eosin (alcohol-soluble)	1 grm.
Absolute alcohol	80 c.c.

SOLUTION C

1 per cent. aqueous solution of hydroquinone.

2. Wash the sections thoroughly in distilled water.

3. Stain for ten minutes in a 1 per cent. aqueous solution of Safranin O of Grübler.

4. Wash thoroughly in distilled water.

5. Treat with 0·5 per cent. potassium bichromate solution for ten to thirty minutes. [An old solution is best.]

6. Wash in distilled water.

7. Dehydrate in absolute alcohol.

8. Clear in bergamot oil. Examine with the microscope ; if the sections contain too much of the safranin, treat them alternately with absolute alcohol and begamot oil until differentiated.

9. Mount in balsam.

Epithelial fibres, red ; nuclei, pale violet ; plasmosomes (true nucleoli), red ; cytoplasm, blue to violet. The granules of neutrophil leucocytes (after formol fixation) are sky blue. Collagen fibres, blue ; elastic fibres, red.

B. *Kromeyer's Method*

This is a modification of Weigert's fibrin stain, and is based

[1] It is often necessary to change the proportions of the three solutions. In such cases you must proceed by the trial-and-error method.

on the fact that the staining reactions of fibrin and of the epithelial fibres (ereidesms) are practically the same.

Fix in absolute alcohol and embed in paraffin. Thin (5 μ) sections are requisite.

1. Stain for ten to fifteen minutes in the following solution :

Methyl-violet 6 B of Grübler Aniline water . . .	Equal parts.

2. Wash well in running water.

3. Treat with dilute Lugol solution (1 part of this to 2 of water) for a few seconds to half a minute.

4. Wash in water.

5. Blot very carefully so as to absorb nearly all the water from the sections.

6. Differentiate the stain in :

Aniline	1 part.
Xylol . . .	2 to 4 parts.

This process must be repeatedly controlled with the staining microscope.

7. Wash the sections in xylol.

8. Mount in balsam.

Epithelial fibres, blue.

C. *Heidenhain's Iron-Hœmatoxylin*

Epithelial fibres are well stained by this technique provided the sections are very carefully destained in the iron-alum solution. A water-immersion objective is very useful here. The fibres are black when properly differentiated.

Cell Bridges (= Deinopores of Shapiro).

When intending to stain these structures, Shapiro advocates that sections be flattened out on the slide with distilled water and *not* with glycerine and albumen. This is because by the latter process a very faintly staining layer of albumen is deposited on the slide, and the delicate spaces in between the bridges may be somewhat obscured by this albuminous coagulum.

Staining by any cytoplasmic stain demonstrates, more or less well, the stained bridges and the unstained intercellular spaces. Shapiro recommends, as giving very clear pictures,

Unna's water blue-orcein-safranin technique (see above) or Heidenhain's iron-hæmatoxylin. In the latter case, differentiation must be very carefully controlled.

Eleidin.—Occurs as intracellular and also intercellular flakes in the stratum granulosum. Eleidin is blackened by osmic acid; it stains intensely with hæmatoxylin and picro-carminate of ammonia (red with latter stain).

The following method demonstrates eleidin:

Make very thin paraffin sections of material fixed in absolute alcohol.

1. Stain for three to eight minutes in very dilute Congo red solution (2 to 5 drops of a 1 per cent. aqueous solution of the dye to 10 c.c. of distilled water).
2. Rinse in water.
3. Stain the nuclei in Delafield or Ehrlich's hæmatoxylin.
4. Blue in running tap water.
5. Dehydrate, clear, and mount in balsam.

Eleidin, red; nuclei and keratohyalin, blue.

NOTE.—Avoid differentiating the hæmatoxylin with any acid solution, as thereby the eleidin is turned blue and is then indistinguishable from the keratohyalin.

Keratohyalin.—The substance characteristic of the stratum lucidum.

Keratohyalin is soluble in concentrated mineral acids, insoluble in ether, chloroform, benzene, and dilute alkalies. But it swells up and dissolves in dilute acetic acid. Fixatives containing this substance (*e.g.* Zenker) are therefore unsuitable for the study of keratohyalin (Weidenreich; Shapiro). It stains with hæmatoxylin of Delafield or Ehrlich, and very intensely with Heidenhain's iron-hæmatoxylin.

The following method is fairly specific for keratohyalin:

1. Overstain paraffin sections in hæmatoxylin of Delafield (or Ehrlich).
2. Rinse rapidly in water.
3. Treat for ten seconds with 0·05 per cent. potassium permanganate or with glacial acetic acid. (The strength of the potassium permanganate solution may need to be increased.)
3. Wash well in water.
4. Dehydrate, clear, and mount in balsam.

Keratohyalin is blue-black. The other elements are relatively unstained.

Keratin.—The substance characteristic of and largely composing the stratum corneum. Keratin resists the action of peptic digestion (although albuminous in nature), water, and most reagents. It stains with great intensity with picric acid. Thus, sections of Bouin-fixed skin, after staining with hæmatoxylin (of Delafield or of Ehrlich) and eosin, still retain a yellow or yellowish tint in the stratum corneum. Specially prolonged treatment with a saturated solution of lithium carbonate in 70 per cent. alcohol is needed to destain keratin. Keratin stains an intense black with Heidenhain's iron-hæmatoxylin, even after full differentiation of the other elements of the epidermis. It is also strongly Gram-positive, and the most definite way of differentiating keratin from other substances in both normal and pathological tissues is to stain sections with Gram's stain (see p. 322) and then treat them with acid alcohol. The stain is thereby extracted from all the elements, except keratin, which remains dark blue.

Mitochondria may be studied by standard methods such as fixation in the fluids of Regaud or Gatenby, and staining by Heidenhain's iron-hæmatoxylin or the Kull technique.

The Golgi Elements are successfully impregnated by Cajal's uranium nitrate-formol method or Da Fano's modification of it.

Dermis.—See also the methods for connective tissue. The most demonstrative sections of the elastic and collagen fibres of this tissue, and their relation to the epidermis, which I have seen were shown me by Dr. Shapiro, who employed a method (unpublished) of E. Fraenkel and communicated by Schmorl.

Fix in formol. Make paraffin sections.

1. Stain in Orth's lithium carmine for twenty to thirty minutes.

Carmine	2·5 grms.
Saturated aqueous solution of lithium carbonate .	100 c.c.

Heat, and then filter.

2. Transfer direct to acid alcohol. Differentiate till only the nuclei are stained.

3. Wash in running water.

4. Stain in the following orcein solution for twenty-four hours :

Orcein *D* (Grübler) . .	1·5 grm.
96 per cent. alcohol. .	120 c.c.
Distilled water . .	60 c.c.
Nitric acid . . .	6 c.c.

Add sufficient of this to a 3 per cent. solution of nitric acid in 80 per cent. alcohol to make it a dark brown colour.

5. Differentiate the orcein in 80 per cent. alcohol.

6. Stain for ten to fifteen minutes in :

Picric acid, saturated aqueous solution	200 c.c.
Indigo carmine.	0·5 grm.

7. Rinse the sections in a 3·5 per cent. solution of acetic acid.

8. 96 per cent. alcohol ; absolute alcohol.

9. Xylol.

10. Mount in dammar or balsam.

Nuclei, red ; elastic fibres, dark brown to almost black ; collagen fibres, green ; muscle, yellow.

EPIDERMAL APPENDAGES

Hair.—The general technique as for skin. If the hair be long it should be cut quite close to the surface of the epidermis before embedding. This is because the hair shafts are apt to split a paraffin ribbon during sectioning. The hair shaft, being mainly composed of keratin, gives the reactions of keratin. In Bouin-fixed material it stains bright yellow. A general method which gives very demonstrative preparations is to section the scalp, in the vertical or horizontal plane, after fixation in Bouin's fluid. Fairly thin sections can be made by using a hard (*e.g.* 55° C. melting-point) wax. Sections are stained by Heidenhain's iron-hæmatoxylin counter-stained in van Gieson or the Ponceau S substitute.

Nails.—Owing to the hardness of these, fœtal material should be taken. The method of fixation is optional, but inclusion in celloidin is a necessity. Or a small fragment of nail and nail-bed may be fixed in formol, embedded in gelatine (see p. 87), and cut with the freezing microtome. Standard staining methods are applicable.

SPLEEN AND LYMPH GLANDS

Most autopsy specimens of these organs are serviceable for general histological observations. The spleen usually suffers from post-mortem deterioration more rapidly than the lymph glands.

An idea of the cellular content of spleen and lymph glands may be obtained from smear preparations. Such specimens are most usefully stained by Leishman's or Giemsa's methods.

For sectional purposes suitable fixatives are :

Mercuric chloride-formol.

Zenker's fluid and Zenker-formol.

4 per cent. formol in 0·9 per cent. sodium chloride.

The fixative chosen should be one (such as the above) which preserves, and permits the differential staining of, red blood corpuscles and leucocytes.

Paraffin sections and standard histological stains can be used. The retiform connective tissue is well shown within sections by Mallory's fuchsin-aniline-blue-orange G stain after fixation in Zenker's fluid. It is stained blue.

SUPRARENAL (ADRENAL) GLANDS AND CHROMAFFIN TISSUE

The suprarenal medulla and the various other deposits of chromaffin tissue (paraganglia ; carotid glands) all possess the property of turning dark brown after prolonged treatment with chromic acid or a chrome salt. This reaction appears to be due to the browning of intracellular adrenalin.

GENERAL METHODS

There are few organs in which autolytic changes occur so soon after death as in the suprarenal glands. The name of " suprarenal capsules " was applied in bygone days to these organs in man simply because all that was left of the tissues of the glands, at the average autopsy, was the capsule enclosing diffluent débris. For all fine histological work it is essential

that they be fixed within an hour or two of death. The ordinary specimens obtained from the post-mortem room are useless for cytological purposes. The glands of small mammals (*e.g.* rats) may be fixed *in toto*. Larger glands should be cut up with a sharp razor. Great care must be taken not to damage the very delicate medulla during manipulation.

Suitable fixatives : mercuric chloride-formol ; 5 per cent. formol ; Zenker's fluid ; Zenker-formol.

Suitable stains : hæmatoxylin of Delafield and the usual counter-stains ; Heidenhain's iron-hæmatoxylin and van Gieson or the Ponceau S substitute.

Demonstration of Intracellular Adrenalin.—See the excellent method of Cramer (p. 163).

Demonstration of Chromaffin Tissue. — The fresher the material the sharper the chromaffin reaction, though staining may be obtained in specimens fixed twenty-four to forty-eight hours post-mortem (Schmorl).

Method I. (recommended by Schmorl).—Fix for two to four days in 2 to 3 per cent. potassium bichromate, to which is added, after half an hour's fixation, 10 per cent. of commercial (40 per cent.) formol. Wash in running water and embed in paraffin. Stain sections in Delafield's hæmatoxylin. Chromaffin cells, yellow to brown. It should be noted that the process of embedding somewhat weakens the chromaffin stain. Frozen sections obviate this.

Method II. (of Wiesel).—1. Fix tissues for one to four days in the following fluid :

5 per cent. aqueous solution potassium bichromate	10 c.c.
10 per cent. formol	20 c.c.
Distilled water	20 c.c.

2. Treat with 5 per cent. potassium bichromate for one to two days.

3. Wash overnight in running water.

4. Dehydrate, clear, and embed in paraffin.

5. Stain sections for twenty minutes in a 1 per cent. solution of toluidin-blue or Wasserblau.

6. Wash in water for five minutes.

7. Stain for twenty minutes in a 1 per cent. aqueous solution of safranin.

8. Treat with 95 per cent. alcohol until the sections turn blue.

9. Clear in carbol-xylol (the ordinary treatment with absolute alcohol followed by xylol does as well).

10. Xylol.

11. Mount in balsam.

Chromaffin cells, green ; nuclei, red ; cytoplasm of cortical cells, light blue.

Method III. (*Giemsa's Stain*).—Good differentiation of medullary and cortical elements may be obtained by Schmorl's adaptation of this stain.

1. Fix material in a formol-bichromate mixture (*e.g.* Regaud's fluid).

2. Wash in running water.

3. Either make frozen sections (said to be preferable), or embed in paraffin.

4. Stain sections for one to twenty-four hours in Giemsa's stain (1 drop of Giemsa for every c.c. of distilled water).

5. Rinse in water.

6. Blot.

7. Dehydrate with acid-free acetone and control differentiation with the staining microscope.

8. Clear in xylol.

9. Mount and examine in cedarwood oil or in balsam. But sections fade in the latter medium.

Medullary cells, bright red to violet ; cortical cells, blue to blue-violet ; red blood corpuscles, pink.

SPECIAL METHODS

The mitochondria may be demonstrated after fixation in Regaud's fluid and staining in Heidenhain's iron-hæmatoxylin. Fixing in the former fluid followed by Kull's stain is also to be recommended. The Golgi elements may be impregnated by the Cajal technique or Da Fano's modification of it.

For the lipoid granules of the suprarenal cortex see Ciaccio's technique (p. 172).

TEETH

The general technique is as for bone (*q.v.*), consequently only certain precautions and special methods are mentioned here.

Freshness of tooth germs seems to be extremely important, as shown by J. T. Carter, if post-mortem changes are to be avoided.

Tooth Germs.—Carter's method is as follows :

1. Fix for five to ten minutes at 37° C. in Sansom's fluid (a modification of Carnoy's fixative). The tooth germs should be fixed in the muco-periosteum after tearing it away from the mandible.

SANSOM'S FLUID

Absolute alcohol	65 c.c.
Glacial acetic acid . . .	5 c.c.
Chloroform	30 c.c.

Add mercuric chloride crystals to saturation.

2. Ascending grades of alcohol from 30 to 100 per cent. The mercury deposit is washed out by the usual iodine treatment during dehydration.

3. Place in equal parts of absolute alcohol and carbon bisulphide for one hour.

4. Pure carbon bisulphide ; two changes each of fifteen minutes.

5. Paraffin plus carbon bisulphide ; thirty minutes at 30° C.

6. Paraffin plus carbon bisulphide ; thirty minutes at 42° C.

7. Pure paraffin ; two changes ; one hour in all.

When decalcification is necessary, Carter uses a 5 per cent. solution of sulphurous acid.

Standard histological stains may be used. Heidenhain's iron-hæmatoxylin gives especially good results.

Adult Teeth. — Most fixatives are suitable. Mummery strongly recommends 33 per cent. formic acid for decalcification. Paraffin is not suitable for large teeth, and celloidin embedding should be used.

Frozen sections furnish preparations with the minimum of shrinkage.

To demonstrate the nerve ends and nerve cells in the pulp, Mummery has devised the following technique :

1. Fix teeth in 4 per cent. formol for a few days. Then increase the concentration to 10 per cent. Leave therein for at least fourteen days. Longer periods do no harm. If the roots are complete, they should be sawn across at the neck to allow the fixative to penetrate.

2. Decalcify in 33 per cent. formic acid to which formol may be added to make up 5 per cent. of formaldehyde. An adult (human) tooth requires fourteen days decalcification.

3. Wash in running water for twenty-four hours.

4. Cut up the teeth into fragments of suitable size and place them in distilled water for a few minutes.

5. Hang the fragments by threads in a 1 in 5000 solution of gold chloride, allowing 100 c.c. of fluid for each fragment. Keep the bottle in the dark and impregnate for from four to seven days.

6. Rinse the tissues in distilled water for a few moments.

7. Place them in 20 per cent. sodium hydrate for four minutes.

(This and stages 9 and 10 comprise the reducing process.)

8. Rinse the fragments in water.

9. Treat with a 10 per cent. aqueous solution of potassium carbonate for one-half to one hour.

10. Transfer the fragments direct to a 10 per cent. aqueous solution of potassium iodide. As soon as the pieces darken (usually after five to ten minutes), transfer them to—

11. Water.

Soak in gum solution for twenty-four hours and make frozen sections. Dehydrate and pass direct from absolute alcohol into camsal (propylic) balsam.

Nerve fibres and nerve cells are impregnated black.

Note.—The 1 in 5000 gold chloride solution should be prepared from a 1 per cent. solution which has been " ripened " by exposure to strong sunlight for a few hours in a glass-stoppered bottle.

TENDONS AND LIGAMENTS

Since these structures are very tough, care must be taken to reduce the duration of fixation, clearing, and embedding (if in paraffin) to the minimum.

Four per cent. formol in normal saline solution is a useful fixative, in that it hardens less than fixatives containing mer-

curic chloride. Paraffin embedding often does not support tendon or ligament sufficiently. In such cases the difficulty can be largely overcome by painting the block with collodion (see p. 83), and cutting on a sliding microtome. To obtain sections across large areas of tendon or ligament, embed in celloidin, which is always preferable for such material where time is not important.

Standard histological stains, and particularly those which demonstrate the different types of connective tissue, may be used. Van Gieson's stain (or the Ponceau S substitute), Weigert's elastin, and Mallory's fuchsin-aniline-blue-orange G method are to be recommended.

THYMUS

Standard histological methods are applicable.

The corpuscles of Hassal are well shown after staining in Heidenhain's iron-hæmatoxylin and van Gieson (or the Ponceau S substitute). Mallory's connective-tissue stain (fuchsin-aniline blue-orange G) is also valuable for this purpose.

THYROID GLAND

Fix in mercuric chloride (with or without formol), 5 per cent. formol, or Zenker's fluid. Make paraffin or celloidin sections.

Good general stains are hæmatoxylin (of Delafield or of Ehrlich) and the usual counter-stains. The colloid inside the vesicles is well shown by Mallory's fuchsin-aniline-blue-orange G stain. For the study of the mitochondria, fix in Regaud's fluid, and stain with Heidenhain's iron-hæmatoxylin or by Kull's method. The Golgi elements may be demonstrated by the Cajal method or by Da Fano's modification of it.

To stain the intracellular colloid droplets use Bensley's technique :

1. Fix in Zenker-formol.
2. Wash for several hours in running water.
3. Dehydrate, clear, and embed in paraffin.

4. Stain thin sections for one or more hours in :

Phosphotungstic acid . .	1 grm.
Brazilin [1]	0·05 grm.
Distilled water . . .	100·0 c.c.

Dissolve the dye in a little warm water. Then add the acid. Ripening can be accelerated by adding 0·4 per cent. of hydrogen peroxide or a few drops of a solution of molybdic acid. The solution should not be kept for longer than three days.

5. Rinse in water.
6. Stain for one to five minutes in :

Phosphomolybdic acid . .	1 grm.
Wasserblau	0·2 grm.
Distilled water . . .	100·0 c.c.

7. Rinse rapidly in water.
8. Dehydrate in absolute alcohol.
9. Clear in xylol or toluol.
10. Mount in balsam.

Nuclei, deep red ; cytoplasm, pink to lilac ; intracellular colloid droplets, deep blue to red ; colloid in thyroid vesicles, blue.

UTERUS AND VAGINA

The uterine epithelium undergoes post-mortem changes (shrinkage and desquamation of its cells) fairly soon after death. Most autopsy material is therefore unsuitable for fine histological observations on the epithelium.

Standard methods of fixation and of staining may be used. The uterine muscle is apt to become very hard during the later stages of dehydration, clearing, and embedding in paraffin. Benzol and oil of cedar harden less than xylol, and should hence be used instead ; the paraffin baths should be as short as possible. A vacuum embedding bath is of use in this respect.

[1] Brazilin, like hæmatoxylin, is an extract from the bark of a tree, the *Cæsalpinia brasiliensis*. Also like hæmatoxylin, it has to be partly oxidised (into brazilein) to get precise staining.

To obtain large sections—*i.e.* across the entire organ or a large segment of it—embed in celloidin.

For "Embryos and Ova," see p. 220.

YOLK GRANULES AND DISCS

See section on these in "Histo-Chemical Methods," p. 183.

PART V

TECHNIQUE OF MORBID HISTOLOGY, INCLUDING METHODS FOR THE IDENTIFICATION OF MICRO-ORGANISMS AND OTHER PARASITES

CHAPTER XV

METHODS FOR HISTO-PATHOLOGICAL PROCESSES

A. General Methods: 1. Fixation—2. Staining.
B. Special Histo-Pathological Methods: 1. Introduction—2. Amyloid Infiltration—3. Calcification—4. Cloudy Swelling—5. Fibrin—6. Fatty Degeneration—7. Hyaline Degeneration—8. Mucoid Degeneration—9. Necrosis—10. Pigmentation—11. Tumours.

A. GENERAL METHODS

INTRODUCTION

Pathological technique, while involving many of the standard histological methods, comprises also a number of staining reactions quite peculiar to it. This is especially so in the case of the methods of staining and identifying of bacteria and spirochætes.

Very broadly speaking, it may be said that the main difference between the methods for the identification of bacteria, and those of normal and morbid histology, lies in the directness—one might almost say the coarseness—of the microscopical part of bacteriological technique. This is due to, and very largely justified by, two considerations: *firstly*, the pathologist of a hospital deals daily with large numbers of examinations. Consequently the simpler the method the better. *Secondly*, bacteria are far more resistant than tissue cells to rough treatment.

Bacteria are divided, from their staining reactions, into two great groups, the Gram-positive and the Gram-negative. Only in the case of certain of them is it possible, by the use of more or less specific staining methods, to determine the species without recourse to methods of cultivation or the injection of animals with the bacterial containing exudate or excretion.

Further, the great extension of serological tests has supplemented or in some instances suppressed the identification of organisms with the microscope.

Only the purely microscopic aspect of bacteriology is dealt with here.

While the methods for the detection of bacteria are thus a mixture of microscopic, cultural, injection, and serological operations, protozoa (and most other animal parasites) are diagnosed, in the laboratory, by methods almost purely histological.

1. Fixation

The following fixatives are suitable for pathological work. The sequence is in order of their utility. Details as to their use are omitted here as they are dealt with in the pages referred to in brackets.

Four per Cent. Formol in 0·9 per Cent. Saline.—The fixative in general use in Great Britain. Not only is the general fixation satisfactory, but tissues and organs may be kept in formol for a considerable period before dehydration, clearing, and embedding are proceeded with. Fat is not dissolved out, although it is in no wise fixed in the sense of being made able to resist the treatment of embedding in paraffin.

Fix tissues for twelve hours or longer.

Bouin's Fluid (formula, p. 35), widely used in France, is another good fixative for post-mortem and pathological material. It has the advantage over formalin that brighter staining with the acid dyes (*e.g.* eosin) is secured by its use.

Fix tissues for twelve hours to three days. (Tissues may be left in this for many weeks without suffering other than a toughening effect.)

Zenker's Fluid (formula, p. 36), employed by Mallory and other pathologists in the U.S.A. Like Bouin, it makes possible brighter staining effects with the acid dyes than formol.

Fix six to twenty-four hours. Tissues fixed much beyond this time are apt to become very hard and brittle.

Chrome-Osmium Fixatives (*e.g.* fluids of Flemming and Champy) are only used, apart from special researches, for the demonstration of fat. Such material can be sectioned by the paraffin method.

Central Nervous System.—See the special section devoted to these methods (p. 233 *et seq.*).

2. Staining

1. Hæmatoxylin (of Delafield or Ehrlich) and Eosin (p. 126).—This is the standard stain for demonstrating the general morbid histology of tissues. It is simple to use and fairly rapid. It is applied as a general routine method of identification to the vast majority of the pathologist's sections. When it is desired to detect bacteria, this method has to be supplemented by one or more of the bacterial stains. (Bacteria are usually but feebly stained by hæmatoxylin—if at all.)

2. Hæmatoxylin and van Gieson (p. 129).—Another important stain for routine identification. The detection of fibrous tissue is often of great importance pathologically.

3. Hæmatoxylin-Erythrosin-Saffron.—This technique of Masson's, described on p. 135, gives an excellent picture of histo-pathological processes.

4. Eosin and Methylene-Blue (p. 133).—More rapid—in expert hands—than hæmatoxylin and eosin. The preparations are also more transparent. Some pathologists—*e.g.* Mallory—employ it as a routine procedure. The preparations are less permanent than with method 1, but it has the advantage of clearly staining in blue most species of bacteria.

5. Heidenhain's Iron-Hæmatoxylin (p. 136), reinforced by a counter-stain such as eosin or the non-fading substitute for van Gieson (p. 129), is the standard method for cytological observations in pathology. It has, furthermore, the advantage over all the above methods in that it is a truly permanent stain. Whence its utility for demonstration specimens and such-like.

For purposes of reproduction, and especially photomicro-

graphy, this stain is, for most tissues, vastly superior to any other.

B. SPECIAL HISTO-PATHOLOGICAL METHODS

1. Introduction

Some important pathological changes are accompanied by physico-chemical alterations in cells and tissues which can be demonstrated by certain special staining methods. Certain of the degenerations and infiltrations fall into this group (*e.g.* hyaline and amyloid change). Certain pathological alterations are quantitative rather than qualitative. Thus, the liver normally contains little intracellular fat. Only when the fat globules attain a size and distribution beyond the normal can fatty degeneration be said to exist. Finally, certain pathological changes have no specific staining reactions; they are recognised partly by their behaviour towards standard stains (*e.g.* hæmatoxylin and eosin), partly by their morphological features. Cloudy swelling (albuminous degeneration) and necrosis are examples.

It must be realised that the earliest appearance of any morbid change which is microscopically detectable is really an advanced or terminal phenomenon from the biochemical standpoint. Cells and tissues, in brief, have to be thoroughly "ill" before microscopic study reveals this fact, and we have every reason to believe that a whole series of complex physico-chemical changes occur before we can diagnose, in the stained specimen, hyaline or an amyloid degeneration.

As in normal, so with morbid, Histology: a given staining reaction, while enabling one roughly (and often conveniently) to diagnose a cell inclusion as cloudy swelling or as hyaline substance, does not usually identify it with any definite chemical substance—except in the rarest instances (*e.g.* glycogen).

Below are arranged, in alphabetical order, the more important of the degenerative and other morbid processes found in tissues. In the case of those which have a histo-chemical counterpart in the normal organism—such as fatty degenera-

tion or calcification—the reader is referred to the Histo-Chemical chapter of this book.

2. Amyloid Infiltration

Amyloid substance is a firm translucent substance. It is always intercellular, and hence is one of the "Infiltrations." Its exact chemical nature is unknown, but it is certain that it is a combination of chondroitin sulphuric acid with an albuminous body.

It is especially a sequel to chronic suppurative and other inflammatory conditions—*e.g.* osteomyelitis, tuberculosis, syphilis, actinomycosis, nephritis, and cancer.

Site.—Especially the spleen ("sago spleen"), liver, and kidneys. Many other tissues may be attacked—*e.g.* adrenals and lymph glands.

Caused by chronic intoxications such as accompany actinomycosis, cancer, chronic nephritis, and syphilis. Also suppurating wounds.

The reactions of amyloid substance are as follows:

Fresh Preparations.—Amyloid substance resists acids and dilute alkalies.

1. Gram's iodine turns amyloid infiltration a deep brown. It is best to add a little dilute acetic acid to the preparation before treating with the iodine solution.

2. Treat first with iodine, then with concentrated suphuric acid. The colour of amyloid material will veer from dark brown to blue.

Sections (Paraffin or Frozen).—The best staining reactions are obtained after fixation in absolute alcohol or a mercuric chloride-containing fixative. The metachromatic reaction with methyl-violet is hampered by prolonged preservation in formol.

1. *Iodine Reaction* . . } Treat as with fresh preparations. Examine sections in water or dilute glycerine.
2. *Sulphuric Acid Reaction* }

3. *Methyl-Violet.*—(1) Stain for two to five minutes in a 1 per cent. aqueous solution of methyl-violet.

(2) Rinse rapidly in water.

(3) Differentiate in 0·5 or 1 per cent. acetic acid, controlling the extraction of the stain with the microscope. When the amyloid substance presents a reddish colour—

(4) Rinse in water.

(5) Mount in lævulose or glycerine jelly.

Amyloid substance, red. Cells and nuclei, in shades of blue. The stain fades in time.

NOTE.—You cannot mount in Canada balsam as the dye is extracted from amyloid substance by alcohol during dehydration.

4. *Congo-Red Method of Bernhold.*—

(1) Stain sections for fifteen to thirty minutes in a 1 per cent. aqueous solution of Congo red.

(2) Treat with a saturated aqueous solution of lithium carbonate for fifteen seconds.

(3) Differentiate in 80 per cent. alcohol.

(4) Dehydrate, clear, and mount (in dammar or balsam). Amyloid substance, bright pink. You can also stain before the Congo-red treatment in hæmatoxylin of Delafield or of Ehrlich. A permanent stain.

NOTES.—(1) Amyloid is metachromatic towards methyl-violet and many other dyes, including gentian violet (amyloid red) and methyl-green (amyloid violet). Metachromasy is discussed on p. 97.

(2) Hæmatoxylin and eosin stains amyloid material a pale pink; with van Gieson it is usually brownish.

3. Calcification

Abnormal deposits of calcareous matter yield, on analysis, approximately the same proportion of calcium salts as normally present in bone—*i.e.* 10 to 15 per cent. of calcium carbonate and 85 to 90 per cent. of calcium phosphate. Consequently the methods are the same for the detection of normal and pathological deposits of calcium.

Calcification of the costal and tracheal cartilages is a normal accompaniment of senility. The deposit of calcium salts in the walls of blood vessels in the later stages of arterio-sclerosis falls partly under this

heading. Calcificatory processes are also prone to occur in any dead or dying area of tissue : the deposit of calcium salts in tubercles and in the bronchial glands of tuberculous subjects are well known. Necrotic adipose tissue, necrotic renal epithelium in cases of poisoning by corrosive sublimate, and necrotic tissues around various parasitic worms—*e.g.* Trichinæ and Cysticerci—are especially prone to impregnation with calcium salts.

Calcification in Living Tissues.—Certain tumours—the osteosarcomata, psammomata, and teratomata—contain deposits of calcium salts.

The microscopical identification of calcium in both fresh preparations and sections is effected by the methods outlined under Histo-Chemical Methods : Calcium Salts, pp. **164** *to* **166.**

4. Cloudy Swelling (= Parenchymatous Albuminous Degeneration)

Cloudy swelling is characterised by an increase in size of both cells and the normal bulk of the affected organ. The cells are crowded with fine granules of an albuminous nature, of complex and unknown chemical nature. The origin of these granules is a debatable point, but there is evidence to show, at any rate in the case of renal epithelium, that the granules are formed by swelling of the mitochondria. Autolysed cells from normal tissue pass through a stage which has been said (Anitschkoff) to resemble cloudy swelling and in which the part played by the mitochondria can be clearly seen. The conclusion to be derived from this is that the fresher the tissues, the better, in the case of organs in which cloudy swelling is suspected.

Distribution : Liver, kidney, striated and non-striated muscle.

A sequel to : Many severe infections and intoxications—*e.g.* diphtheria, pneumonia, or typhoid.

Fresh Preparations.—The diagnosis of cloudy swelling can be rapidly made from such preparations.

1. Add 2 per cent. acetic acid to the preparation.
2. Add 2 per cent. NaOH or KOH to the preparation.

} The droplets are dissolved in the case of cloudy swelling.

3. The granules are insoluble in the standard fat solvents (ether ; chloroform ; absolute alcohol).
4. The granules are not blackened by osmic acid of 1 or 2 per cent.

} These tests serve to differentiate the intracellular granules of cloudy swelling from those of fatty degeneration.

Usually test No. 1 suffices for the identification of cloudy swelling.

Sections.—There is no specific histo-chemical method for demonstrating cloudy swelling.

Hæmatoxylin and eosin or eosin-methylene-blue sections show the swollen and diffusely staining cells. In the case of renal epithelium, the unattached poles of the cells are irregular and may obliterate the lumen of the tubule. The nucleus usually stains more faintly than in normal tissues.

5. Fibrin

Fresh Preparations.—Add 0·5 per cent. acetic acid. Fibrin swells up and rapidly dissolves.

Sections.—*Weigert's Fibrin Stain.*—This method, which is really a modified Gram's stain, is by no means specific for fibrin, since hyaline degeneration is sometimes stained by it. So are Gram-positive bacteria. The stain is also easily lost by fibrin if differentiation be too long. The identification of fibrin is thus partly morphological, partly based on its reaction to this stain. Fix (preferably) in absolute alcohol. Make paraffin sections. Treat sections on the slide as follows :

1. Stain for three to five minutes in *either* a 1 per cent. aqueous solution of methyl-violet *or* in aniline water-gentian violet (formula, p. 116).
2. Rinse in water.
3. Treat with Gram's iodine (formula, p. 117) for three to five minutes.
4. Blot thoroughly with filter paper.
5. Decolorise until the fibrin is blue but the tissues colourless (or nearly so) in :

Aniline oil . . }
Xylol . . . } Equal parts.

6. Carefully blot so as to remove the aniline xylol.
7. Rinse in water.
8. Stain in a 1 per cent. aqueous solution of neutral red for one to two minutes.
9. Rinse rapidly.
10. Carefully blot up the water.
11. Dehydrate, clear and differentiate the neutral red in aniline oil. When the nuclei appear a bright pink, transfer to :
12. Xylol—to arrest differentiation.
13. Mount in dammar or balsam.

Fibrin, blue-black ; tissue constituents, in shades of red ; Gram-positive bacteria, blue-black ; Gram-negative bacteria, pink. Hyaline degeneration is sometimes stained like fibrin.

NOTES.—The differentiation of the stain for fibrin (stage 5) and of the neutral red (stage 11), must be controlled with the staining microscope. Great care must be taken not to destain the fibrin during stages 5 and 11.

Mallory's connective-tissue stain, provided the tissues have been fixed in Zenker's fluid, also enables one to identify fibrin formations in that they are stained *red.*

6. Fatty Degeneration

By fatty degeneration is meant either the appearance of microscopically detectable fat in tissues normally devoid of it, or its excessive increase in amount in tissues which normally contain but little. The question of "masked" fat—already referred to on p. 167—must be kept in mind when examining preparations treated by the fat stains. The normal distribution of microscopically discernible fat has been dealt with on p. 9.

DISTRIBUTION.—Fatty degeneration may attack almost any of the organs or tissues. In very obese persons there occurs, in addition to an enormous development of fat stores in connective tissue, a deposit of fat in cells normally devoid of it, or only containing it in moderate quantities. The liver cells, for instance, often participate in the fatty change. Apart from obesity, nearly all the infections and intoxications can, and frequently do, produce fatty degeneration, especially in the liver, kidneys, and heart muscle.

Caused by a variety of conditions, chief amongst which are: Defective nutrition (*e.g.* starvation) or diminished activity (*e.g.* paralysis of muscles); toxins of diphtheria, pneumonia, puerperal infection, scarlatina, tuberculosis, and typhoid. Frequently associated with amyloid infiltration. Poisoning by alcohol, antimony, arsenic, chloroform, phosphorus. Also pernicious anæmia and diabetes.

FRESH PREPARATIONS
SECTIONS . . . } The technique is identical with the tests for normal fat described on pp. 169 to 173. For differentiation between the granules of cloudy swelling and fat globules, see p. 309.

FAT EMBOLI.—Fix small pieces of tissue in Flemming's fluid. After embedding in paraffin, sections can be stained (if desired) in safranin or eosin-methylene-blue.

Or you can fix in 5 per cent. formol in 0·9 per cent. NaCl, cut on the freezing microtome (in the case of lung, inclusion in gelatine is advisable), and stain in Scharlach R or Sudan III. (see p. 170).

7. Hyaline Degeneration

By hyaline degeneration (or substance) is meant a refractile, shiny substance (in the fresh condition). In staining reaction it is oxyphil towards the standard dyes. Chemically nothing is known regarding it beyond that it is proteid in nature. Cells which have undergone the hyaline change are dead and often pass first of all through necrotic and other phases. The group of hyaline substances is still a very vaguely defined one.

DISTRIBUTION.—Hyaline transformation of the walls of the blood vessels in the senile uterus and ovary is the rule. The final stage of the corpus luteum is also a hyaline conversion of its scar tissue.

Hyaline degeneration can appear in a variety of tissues: the liver, skeletal and cardiac muscle, the renal epithelium (whence the hyaline casts of urine), thrombi, and connective tissue are prone to it. The connective-tissue stroma of lymph glands and of malignant tumours may also undergo this change.

A sequel to many acute infections—tuberculosis, typhoid, scarlatina, and diphtheria—and old suppurating wounds.

FRESH PREPARATIONS.—Hyaline substance may be amorphous or in the shape of intracellular granules.

Very refractile ; resists dilute acids and alkalies and alcohol ; unaffected by water.

It does not give the dark brown iodine reaction of amyloid infiltration, nor does hyaline material so treated turn blue after treatment with sulphuric acid.

SECTIONS.—Most fixatives can be used except those containing osmic acid.

1. *Kühne's (Modified) Technique.*—The method of fixation is optional. (1) Stain paraffin sections on the slide for three minutes in the following (freshly mixed) : a saturated solution of crystal violet in absolute alcohol, 9 parts ; 1 per cent. aqueous solution of ammonium carbonate, 1 part.

(2) Rinse in tap water.

(3) Treat with Lugol's solution (formula, p. 117) for two minutes.

(4) Rinse rapidly in tap water.

(5) Differentiate in a saturated solution of fluorescein in absolute alcohol until the hyaline material is stained in various shades of blue, and the other tissues decolorised.

(6) Rinse in tap water.

(7) Counter-stain for two minutes in a 1 per cent. aqueous solution of neutral red.

(8) Rinse in water.

(9) Dehydrate rapidly in 90 per cent. and absolute alcohol, controlling the degree of differentiation of the neutral red with the staining microscope.

(10) Clear in xylol, and mount in dammar or balsam.

NOTE.—The introduction of the neutral red into this method gives an extremely transparent nuclear stain, while the hyaline substance is stained in shades of blue—the older the hyaline, the darker the blue.

2. *Russel's Carbol-Fuchsin Method.*—This method gives good results after any of the standard fixatives. Embed in paraffin and treat sections on the slide as follows :

(1) Stain in carbol-fuchsin (formula, p. 116) for ten to thirty minutes.

(2) Rinse in water.

(3) Pass rapidly (one-half to one minute) through absolute alcohol.

(4) Differentiate and counter-stain for a few minutes in the following solution :

Iodine green	1 grm.
5 per cent. aqueous solution of phenol .	100 c.c.

(5) Rinse rapidly in absolute alcohol.

(6) Clear in xylol.

(7) Mount in dammar or balsam.

Hyaline substance, bright red ; nuclei, light green.

3. *Weigert's Fibrin Stain* (see p. 310).—This stains hyaline material like fibrin—*i.e.* blue-black.

4. *General Histo-Pathological Methods.*—These do not give any specific stain for hyaline material :

Hæmatoxylin and eosin : hyaline substance, pink.

Eosin-methylene-blue : hyaline substance, pink.

Hæmatoxylin and van Gieson : hyaline substance, yellow to light red.

In brief, the general reaction of hyaline material is oxyphil.

8. Mucoid Degeneration (= Myxomatous or Gelatinous Degeneration)

See section on Mucin, p. 176.

The appearance of substances belonging to the mucin group in tissues normally devoid of it is known as mucoid degeneration.

Sites.—Bone marrow ; connective tissue ; mucous polypi of the nose ; tumours (chondromata, fibromata, lipomata, sarcomata, etc.).

Accompanying : Senility or alcoholism (mucoid transformation of bone marrow) ; myxœdema (connective tissues generally) ; various chronic inflammatory conditions ; as a degenerative phenomenon in the above-named tumours it is fairly common.

Identification of Mucoid Degeneration.—

Fresh preparations . } As for Mucin (pp. 176 and 177).
Sections . . }

9. Necrosis

Areas of dead tissue—unless they can peel away, as on a free surface—undergo one of two fates : *either* they are removed—partly by solution by autolytic ferments, partly by

mechanical removal through phagocytes—*or* they undergo necrosis. By this is meant the gradual disintegration of nuclei and cell outlines. The most specialised components of the cytoplasm (*e.g.* myofibrillæ) are the first to disappear; next the nucleus, after preliminary degenerative phenomena; finally, the cell outlines and the general architecture of the tissues are lost as they become changed into a more or less granular substance. Sometimes fragmented nuclei can be found in such areas, which are said to have undergone "*Caseation*." Caseation, which is the last word in tissue degeneration, is most frequently the end-result in the unhealed tubercle.

Any cause or agent which kills tissues will produce necrosis. Thus, obstruction of the blood supply, the action of acids or alkalies, or of extreme heat or cold, or very powerful toxins—these and many other factors will cause necrosis.

Identification of Necrosis.—From the above, it follows that the microscopical recognition of necrosis is mainly morphological. Diffuse or abnormal staining of tissues smitten with this change is the rule.

The sectional method is best for studying necrosed tissues. Fixation in a standard fixative, followed by staining with Delafield's or Ehrlich's hæmatoxylin and eosin, or eosin-methylene-blue, will reveal necrosed areas.

Weigert's fibrin stain frequently stains strands or granules of a substance present in the later stages of necrosis.

10. Pigmentation

See the methods for the histo-chemical identification of pigments (pp. 178 to 183).

Hæmoglobin and its derivatives are found in tissues adjacent to any area in which blood cells have been destroyed on a large scale.

The iron-free pigments—when derived from hæmolysis of red blood corpuscles—are found under similar conditions.

Melanin of the skin is increased in certain morbid conditions—*e.g.* in exophthalmic goitre and Addison's disease. The cells of the melanotic sarcoma are packed with it.

The coloured fatty pigments — the lipochromes — are

especially found in certain tumours (xanthomata and chloromata).

Pigment of extraneous origin, not formed by the cells themselves, is found in the pneumoconioses, and also in lead and silver poisoning (plumbosis and argyria).

11. Tumours

The general technique is largely that of ordinary Histology. Useful fixatives are :

4 or 5 per cent. formol in isotonic saline ;
Mercuric chloride-formol (formula, p. 37).
Bouin's fluid (formula, p. 35).
Zenker's fluid (formula, p. 36).

Removal of the Tissues.—Pieces of tissue in which cancerous changes are apprehended should be placed immediately after excision in a tube of the fixative. An account of the site and suspected lesion should always accompany the specimen. Furthermore, the specimen, whenever possible, should comprise a segment of normal tissue, so that any phenomena of invasion—of fundamental diagnostic importance—may be registered. Thin slices of tissue of as large an area as possible are the most suitable. If the suspected lesion is not homogeneous, it is better to excise several portions of it. The avoidance of crushing the tissues during removal must be observed. If no fixative is available do not place the specimen in an empty bottle, or in saline or water. Maceration and autolysis are rapidly induced by such treatment. The best plan in such cases is to wrap the pieces of tissue in oiled silk or some other non-absorbent substance. Then place them in as cool a place as possible. The day should have passed for the sending of lumps of unfixed, macerated, and autolysed tissues for examination.

As a substitute for proper fixation, tissues may be placed in methylated spirit or, better, in ethylic alcohol of 60 to 70 per cent.

Biopsy Material.—Wherever possible, such fragments should be fixed immediately. In some cases, as in the removal of fragments of urinary papillomata and in uterine

curettings, damage to the tissues during extraction is inevitable. All the more care is therefore needed in the microscopic interpretation. It may even be necessary to make serial sections or to take isolated ones at various levels right through the fragment, in order to reconstruct the relationships of the tissues. In the case of uterine curettings all the shreds should be sectioned, owing to their fragmentary nature.

Staining Methods.—For routine work, paraffin sections stained by hæmatoxylin (of Delafield or of Ehrlich) and eosin are useful. Masson's hæmatoxylin-erythrosin-saffron stain (see p. 135) gives very clear pictures of neoplasms. Fibrous tissue is demonstrated by standard methods. In the case of gliomata it may be necessary to apply a specific stain for neuroglia fibres. The methods of Lhermitte and Guccione (p. 264) or of Anglade and Morel (p. 266) are of use in this respect.

Rapid Examination of Tumours.—If a report is needed in a few hours, Masson's method is very good.

1. Fix very thin (1 to 2 mm. thick) pieces of tissue for thirty minutes in the fixative of Duboscq-Brasil (an alcoholic Bouin).

80 per cent. alcohol . .	150 c.c.
40 per cent. formol . .	60 c.c.
Glacial acetic acid . .	15 c.c.
Picric acid crystals . .	1 grm.

2. Absolute alcohol (two baths), forty minutes.
3. Toluol (two baths), forty minutes.
4. Paraffin (two baths), forty-five minutes.
5. Casting and embedding, ten minutes.
6. Sectioning, fifteen minutes.
7. Drying the sections on the hot plate, fifteen minutes.
8. Staining, fifteen minutes.
9. Examination of the sections, thirty minutes.

Total time required, four hours.

Stages 6, 8, and 9 may usually be abbreviated and the time still further reduced. The quality of the sections obtained is quite good, and amply sufficient for all diagnostic purposes.

Methods suitable for Use at Operation.—Sometimes the surgeon, after exposing a tumour, excises one or more

portions which are immediately examined for the presence or absence of malignancy. In certain operations, and especially in doubtful cases of breast cancer, the patient may be spared the disfigurement of the radical operation by a favourable histological report. The first requirement in all such methods is speed. The presence of a thoroughly competent pathologist is essential, and the value of these methods is in strict relationship to his capacity.

Frozen Sections.—1. Fix thin slices of tissue (2 to 5 mm. in thickness) in hot formol of 5 to 10 per cent. for a few minutes.

2. Section with the freezing microtome.

3. Stain in a watch-glassful of a rapid stain such as Delafield's hæmatoxylin, carbol-thionin, or methylene-blue.

4. Mount in dilute glycerine or water.

Note.—Such sections are in no wise permanent, as they lose their colour rapidly in aqueous mounting media. But this does not matter, since the only aim of these rough methods is an immediate diagnosis.

Champy's Method.—This technique has been used in doubtful cases in the Gynæcological Service at the Faculty of Medicine of Paris.

1. The tumour having been exposed at operation, drop one or (preferably) more fragments about 1 cm. square into the following fixative in a large test-tube :

Absolute alcohol . .	3 parts.
40 per cent. formol .	1 part.

Boil the tissues gently for two minutes in this.

2. Make freehand sections with a good razor. If preferred, you may hold the fragment of tissue between two pieces of pith.

3. Transfer the sections direct to concentrated Ehrlich's hæmatoxylin (Delafield's hæmatoxylin is even better) for thirty seconds. This and all subsequent solutions should be previously prepared in watch-glasses.

4. Transfer to a saturated aqueous solution of picric acid for a few seconds only.

5. 95 per cent. alcohol.

6. Absolute alcohol.

7. Xylol.

8. Mount in clove oil, cover, and examine.

Nuclei, red ; other elements, yellow.

Total time required to prepare the sections, four to eight minutes per block of tissue.

NOTE.—The majority of the sections are very thick. This does not matter, provided you know how to interpret thick sections. As pointed out by Champy, this is easily attained by the study of thick sections of museum specimens.

CHAPTER XVI

BACTERIOLOGICAL METHODS

1. Preparation of Films and Smears—2. Fixation—3. Staining Films and Sections: (i) Nicolle's Carbol-Thionin; (ii) Gram's Method; (iii) Claudius' Method; (iv) Ziehl-Neelsen Technique; (v) Preparation of Sputum for Microscopical Examination: (*a*) Standard Method; (*b*) Antiformin Method—4. Table of Gram-positive and Gram-negative Pathogenic Micro-Organisms.

THE introductory remarks to "Pathological Methods" are largely applicable here. There is a table of the staining reactions of micro-organisms towards Gram's stain at the end of this chapter.

1. THE PREPARATION OF FILMS AND SMEARS FOR BACTERIOLOGICAL EXAMINATION

Have ready some perfectly clean slides. In the case of a fluid which is suspected to contain micro-organisms, place a drop of it on the slide and gently smear it with the platinum loop. Either allow to dry or warm it very gently. In the case of a centrifugalised fluid, such as urine, pour off the supernatant fluid and take up on to the slide a drop of the remaining liquid (or sediment) with a platinum loop. Bacteria from cultures, or solid or semisolid substances, are also taken up with the platinum loop.

NOTE.—For making smears of sputum, see p. 328.

2. FIXATION

Fixation of the film may be effected, once it is dry, by slowly passing the slide, film upwards, through the flame of the Bunsen burner three times. This method, though widely used, is NOT to be recommended. It is far better to fix in absolute alcohol, or in absolute alcohol and ether, equal parts. A thin

film is fixed practically instantaneously, a thick one in two or three minutes, and the distortion of cells produced by the heat-fixation technique is avoided. Keep a tube of alcohol, or of ether-alcohol, and fix slides by immersion in it.

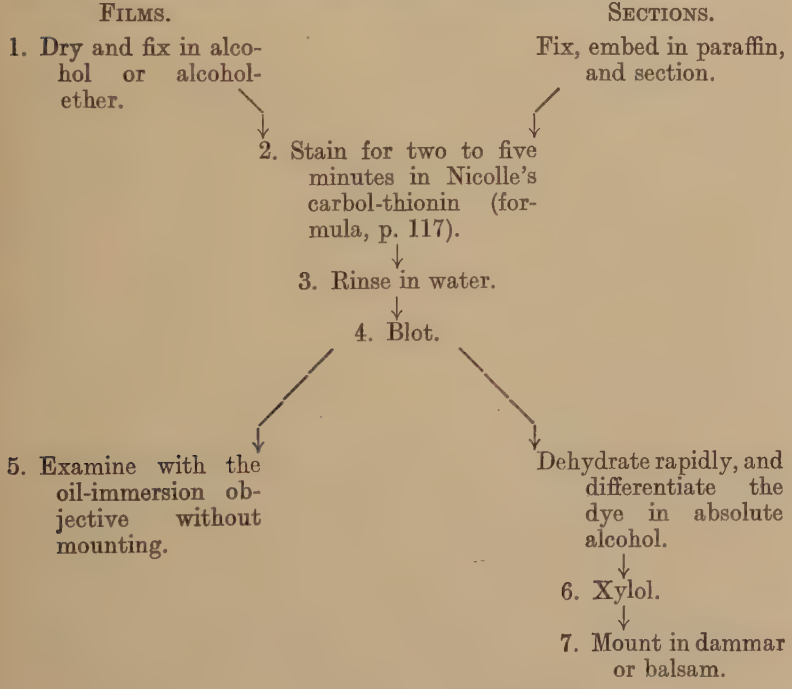
The smear is then ready to be stained.

Fixation of tissues for sectioning is usually done with formol of 4 to 5 per cent. in 0·9 per cent. NaCl. But the mercuric chloride-containing fixatives mentioned on p. 37 are also very useful. Dehydrate, clear, and embed in paraffin according to the standard technique.

3. STAINING FILMS AND SECTIONS

(i) Type Method: Nicolle's Carbol-Thionin

This method is chiefly useful for the rapid staining of films or smears from cultures or organs. Also useful for ascertaining the presence of bacteria in sectioned tissues.


Films.
Sections.
1. Dry and fix in alcohol or alcohol-ether.
Fix, embed in paraffin, and section.
2. Stain for two to five minutes in Nicolle's carbol-thionin (formula, p. 117).
3. Rinse in water.
4. Blot.
5. Examine with the oil-immersion objective without mounting.
Dehydrate rapidly, and differentiate the dye in absolute alcohol.
6. Xylol.
7. Mount in dammar or balsam.

Bacteria, deep blue ; cells and nuclei, in lighter shades of blue ; mucin, granules of mast cells (if preserved), and the ground substance of cartilage, purple.

NOTES.—(1) Films generally do not require any differentiation. Should this be necessary, treat exactly as for a section, but blot after xylol instead of mounting in balsam.

(2) Thionin fades in course of time if the preparation be mounted.

(3) Leishman's blood stain (p. 205) is very useful for demonstrating cells and bacteria (the latter dark blue), in films of cell-containing fluids (*e.g.* urine, pleural, and other exudates).

(ii) Gram's Method

This is the classical staining method of the bacteriologist. By means of it micro-organisms are divided into two groups, the Gram-positive, which retain the stain, and the Gram-negative organisms, which lose it. Further, it is sometimes possible to distinguish, by their opposed staining reaction, two species of bacteria which are morphologically similar. The method is a regressive one, in that both Gram-positive and Gram-negative bacteria are first stained in a powerful basic aniline dye (methyl-violet or gentian violet), then treated with iodine and finally differentiated until only the Gram-positive organisms retain the stain. In order to see Gram-negative bacteria, a counter-stain (neutral red, carbol-fuchsin, or Bismarck brown) is usually applied after differentiation of the Gram stain, though this did not form a part of the original method.

There are two explanations for the intensive staining of Gram-positive organisms. One is that the iodine acts as a mordant towards the dye on the one hand, and the protoplasm of Gram-positive organisms on the other : it links the former to the latter. The other explanation is that the iodine, by penetration into the protoplasm of the Gram-positive bacteria, precipitates the stain therein ; whence the enhanced resistance of the stain towards the differentiating action of alcohol. Both views are based on a physical or chemical difference in the substance of Gram-positive and Gram-negative bacteria.

Type Method: Gram-Neutral Red (or Carbol-Fuchsin)

FILMS.

1. Dry and fix in absolute alcohol or ether-alcohol.

SECTIONS.

Fix, embed in paraffin, and section. Then remove paraffin with xylol and pass through absolute alcohol to water.

↓

2. Stain in carbol-gentian violet[1] *or* in 1 per cent. aqueous methyl-violet for two to three minutes.

↓

3. Wash off the stain with Gram's iodine; then flood the slide with the same; leave for two to three minutes.

↓

4. Decolorise the film or section in absolute alcohol (*see Note* 1).

↓

5. Rinse rapidly in water.

↓

6. Stain for two to three minutes, *either* in a 1 per cent. aqueous solution of neutral red *or* for thirty to sixty seconds in carbol-fuchsin (formula, p. 116), *diluted* in the proportion of 1 to 10 (of distilled water).

↓

7. Rinse rapidly in water.

↓

8. Blot and dry. Examine with the oil-immersion lens without mounting.

Dehydrate very rapidly (and differentiate the counter-stain) in absolute alcohol.

↓

9. Xylol.

↓

10. Dammar or balsam.

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[1] Gentian violet . . . . 1·0 grm.
Phenol cryst. . . . . 2·0 grms.
Absolute alcohol . . . 10·0 c.c.
Aq. dest. . . . . 100·0 c.c.
Mix and leave for twenty-four hours; then filter.

Gram-positive bacteria, blue to black; Gram-negative organisms, red. In the case of sections the tissues are pink; with the dilute Ziehl-Neelsen stain they are rather diffusely stained, but with neutral red the histological details are better.

NOTES.—(1) *Gram's stain is easily under- or over-differentiated.* In the former case, Gram-negative bacteria are stained blue, in the latter, even Gram-positive organisms may give a red (Gram-negative) stain. Only experience and practice will enable you to "hit off" the right degree of differentiation.

(2) When in doubt as to the staining reaction of organisms the following is of use: Make a small film from a culture of a well-known Gram-positive and a Gram-negative organism on the same slide as your smear or section. For instance, a *Staphylococcus* and *Bacillus coli communis.* Fix these films in absolute alcohol at the same time as you fix the smear, or, in the case of a section, treat it in the following sequence: absolute alcohol (to fix the control film); xylol (to remove the paraffin); absolute alcohol (to remove the xylol). Then stain as above. Such controls are sometimes very useful in cases of doubtful staining.

(3) *Bear in mind that the following organisms are only stained after prolonged staining* (e.g. *twelve to twenty-four hours*) *by the Gram method* (*and its modifications*—e.g. *Claudius*) *: Bacillus tuberculosis, and Bacillus of malignant œdema.*

(4) In sections which have been counter-stained so as to demonstrate both Gram-positive and negative bacteria, it frequently happens that Gram-positive organisms, if scanty and lying inside cells (which are Gram-negative in reaction), stain as the surrounding tissues—*i.e.* Gram-negatively—owing to the influence of these on the bacteria.

(5) The staining reactions of organisms from old cultures are not trustworthy.

(iii) Claudius Modification of Gram's Stain

This method has the great advantage over the original Gram technique in that Gram-positive bacteria cannot be decolorised even by prolonged differentiation. It is to be strongly recommended, and the following modification gives reliable results:

Films.	Sections.
1. Dry and fix in absolute alcohol or ether-alcohol.	Fix and embed in paraffin. Bring down to water.

2. Stain with *either* 1 per cent. aqueous methyl-violet *or* carbol-gentian violet (formula, p. 323) for one or two minutes. (Claudius recommends "methyl-violet 6 B extra" of Merck.)

3. Rinse in tap water.

4. Pour on to the slide a half-saturated aqueous solution of picric acid. Leave two to three minutes.

Blot thoroughly.

5. Decolorise with picric acid-aniline oil [1] until no more blue washes out from the film or section. In the latter case this may take fifteen to thirty minutes.

6. Blot thoroughly to remove the aniline oil.

7. Rinse in water.

8. Counter-stain Gram-negative organisms and the tissues for one to three minutes in a 1 per cent. solution of neutral red.

9. Rinse rapidly in water.

10. Blot thoroughly.

[1] *i.e.* Aniline oil, to which 0·1 per cent. of picric acid is added.

11. Dry in air. Examine without mounting with an oil-immersion lens.

Dehydrate very rapidly in pure aniline oil, or in absolute alcohol, controlling at the same time the extraction of the neutral red. When the sections are a transparent pink, clear in—

↓

12. Xylol.

↓

13. Mount in dammar or balsam.

The staining is the same as for a Gram-neutral red. Notes 2 to 5 of the preceding technique apply to this method.

NOTE.—Clove oil or chloroform may be used for decolorising in place of the picric-aniline oil. Claudius used the former; but it is a costly reagent.

(iv) The Ziehl-Neelsen Technique

This, or one of its modifications, remains the standard method for staining tubercle bacilli. The bacillus of leprosy is also stained by it.

The Ziehl-Neelsen method is based upon the following peculiarity of these two organisms: When bacteria of any sort are placed for a sufficient time in carbon-fuchsin they all stain deeply. But if the film or section be treated with sulphuric acid of 25 per cent. (or less), or with absolute alcohol, the bacteria all lose their colour with the exception of *Bacillus tuberculosis* and *B. leprœ*, which withstand both acid and alcohol for far longer than the other bacteria. Whence the terms "acid-fast" and "alcohol-fast" often applied to these organisms. Their resistance to extraction of the stain is probably due to their waxy envelope. In order to see organisms other than *B. tuberculosis* and *B. leprœ*, the film or section is usually counter-stained with a basic aniline dye—*e.g.* methylene-blue.

FIXATION OF TISSUES AND STAINING WITH ZIEHL-NEELSEN

It is advisable to fix tissues for examination for tubercle bacilli in mercuric chloride, and NOT in formol which is said to

inhibit staining of tubercle bacilli by the Ziehl-Neelsen technique.

Smears.

1. Dry and fix in absolute alcohol or ether-alcohol.

Sections.

Fix in mercuric chloride in saturated aqueous solution. Embed in paraffin.[1]

2. (*a*) *Either* flood the slide with carbol-fuchsin (formula, p. 116), after placing a rectangle of filter paper (to prevent precipitation of the stain) over the film or section. Hold above Bunsen burner until the stain just steams; then put aside for five minutes.

Or

(*b*) Place in a vessel of carbol-fuchsin for one-half to one hour at 37° C. (*e.g.* in the incubator).

3. Rinse in water.

4. Decolorise with 25 per cent. sulphuric acid for three to five seconds.

5. Wash in 60 per cent. alcohol till no more stain comes away.

6. Rinse in water.

7. Counter-stain in Borrel's methylene-blue (formula, p. 110), diluted in the proportion of 1 to 5 of distilled water, for one-half to one minute.

Decolorise in 1 per cent. HCl in 70 per cent. alcohol for one minute.

Continue the decolorisation in neutral 60 or 70 per cent. alcohol for two to three minutes, until the section is a very pale pink.

Rinse in water.

Counter-stain in Borrel's methylene-blue diluted in the proportion of 1 in 5 of distilled water, for one to two minutes.

[1] If the material has been fixed in formol, staining of tubercle bacilli is facilitated by treating the sections, before staining, with 0·5 per cent. ammonia for a few minutes.

8. Rinse in water.
↓
9. Blot and dry.
↓
10. Examine with the oil-immersion objective without mounting.

Rinse in water.
↓
Dehydrate and differentiate the methylene-blue in absolute alcohol.
↓
Xylol.
↓
11. Mount in dammar or balsam.

Tubercle (and leprosy) bacilli, bright red ; other bacteria, blue ; cells, cell débris, and mucin in sputa, in shades of blue ; in sections the cell nuclei, etc., are blue. The fuchsin component does not fade, but the methylene-blue weakens somewhat in course of time, owing to the balsam going acid.

Notes.—(1) If Ziehl-Neelsen is used to demonstrate the leprosy bacillus, the differentiation of the fuchsin must be carefully done, as this bacillus is more easily completely decolorised than *B. tuberculosis*. In the case of films, sulphuric acid of 5 per cent. (in lieu of 25 per cent). is indicated.

(2) Since the methods indicated above both involve the use first of acid, then of alcohol, the risk of mistaking the smegma bacillus (which often is acid-resistant, but rarely both acid- and alcohol-fast) for *B. tuberculosis* is diminished. This remark also applies to various other acid-fast bacilli—*e.g.* those contained in milk, butter, and the cerumen.

To make quite certain of decolorising smegma, alcohol treatment should be prolonged for three to twelve hours when smegma is suspected.

(v) The Preparation of Sputum for Microscopical Examination

(*a*) Standard Method

Take up a little sputum with the platinum loop ; in tuberculous sputa the greyish particles are best. Place the material on a slide, then pressing another slide down flat upon the sputum, pull away the slides, one of which is held in each hand. Repeat this process of pressing and pulling until you

have a thin layer of sputum on each slide. Such a smear should extend from one extremity of the slide to about ¾ inch from the other end. This, the sputum-free end, is used for manipulation, and can be marked with an index number with the grease pencil. Then stain as above.

Note that the above method, while excellent for bacteria, greatly deforms tissue cells and leucocytes. For observations on these, the slide must be lightly smeared with a platinum loopful of the material.

(*b*) The Antiformin Method

Frequently it is impossible to detect tubercle bacilli in sputum even after repeated examination by the above method. In such cases a positive observation may often be achieved by treatment of the sputum with chemical agents capable of dissolving its mucin, cells, and cell débris. A fluid—instead of a viscous mass—is thus obtained, the deposit of which can be stained and microscopically examined after centrifugalisation. Tubercle bacilli, on account of their waxy envelope, resist the solvent action of the antiformin (or its substitute).

Calmette's Modification of the Antiformin Technique of Uhlenhuth and Xylander

A stock solution of antiformin, 30 parts, and distilled water, 100 parts, is kept in the laboratory. (Antiformin is a disinfectant, commercially produced, composed of equal parts of liquor sodæ chlorinati and 15 per cent. aqueous solution of sodium hydrate.)

Note.—The distilled water should be freshly distilled and not taken from an old stock-bottle of distilled water, since this sometimes contains acid-fast bacilli.

1. Mix in a centrifuge tube (the larger the better) equal volumes of sputum and of the stock antiformin solution. Shake vigorously.

2. Place in the incubator at 37° C. for two to three hours. At 60° C. (*e.g.* in the paraffin thermostat) one-half to one hour is sufficient.

3. Centrifugalise.

4. Decant the supernatant fluid, fill up with 0·9 per cent. saline, and shake.

5. Centrifugalise again.

6. Pour off the supernatant fluid and make films on slides from the deposit with the platinum loop.

These films are then stained by the Ziehl-Neelsen method (p. 327).

NOTES.—(1) If a centrifuge is not available, Jacobson's ligroin-antiformin method is useful. Proceed with stages 1 and 2 as above; then add ligroin (petroleum ether) so as to form a layer about 3 mm. thick above the antiformin-sputum mixture. Shake and incubate again for half an hour. Then carefully aspirate with a pipette the greyish layer (containing the bacilli) which is formed at the interface between the antiformin-sputum mixture and the supernatant ligroin. Stain films made from this layer by the Ziehl-Neelsen method.

(2) The antiformin method is not only applicable to sputum; it is applicable to all fluids containing many cells (*e.g.* urine) and also fæces (in suspected cases of alimentary tuberculosis). It may even be applied to sections (Merkel), in which case thick sections of unfixed material should be cut with the freezing microtome (for technique, see p. 86), and then treated according to the standard antiformin technique described above. The centrifugalised deposit, in such cases, should be fixed to slides by the glycerine and albumen method as follows: Smear the surface of the slide with a very thin layer of the concentrated glycerine and albumen mixture (see p. 77). Add the deposit to this. Then dry on the hot plate for half an hour or longer.

(3) Antiformin treatment does not kill tubercle bacilli unless very prolonged. Consequently centrifuge tubes, etc., should be sterilised after being used for material containing suspected tubercle bacilli. But bacteria other than the acid-fast ones are dissolved by antiformin.

(4) Precautions with regard to the smegma bacillus being mistaken for *B. tuberculosis* are only necessary (*a*) in the case of urine when not taken aseptically with a catheter (especially in the case of women); and (*b*) if the combined treatment first with acid and then with alcohol is not adhered to. (In the Ziehl-Neelsen technique, as originally described, only decolorisation in acid is prescribed for films.) See Note 2 on p. 328.

4. Table of Gram-positive and Gram-negative Pathogenic Micro-Organisms

The following facts must be borne in mind when staining bacteria by the Gram method or by its modifications (e.g. *Claudius*) *:*

1. Certain bacteria are only Gram-positive after prolonged staining (see under).

2. Bacteria taken from old cultures often stain atypically ; particularly is there a tendency for Gram-positive organisms to be Gram-negative.

3. When staining bacteria in tissues it sometimes happens that Gram-positive organisms stain as the tissues—*i.e.* Gram-negatively. The appropriate means for testing a case of doubtful staining is indicated on p. 324.

4. Although the general reaction of tissues is Gram-negative, it must be remembered that areas of keratinisation or (sometimes) calcification, and also fibrin, are Gram-positive.

Gram-Positive Organisms.	Gram-Negative Organisms.
Bacillus of anthrax.	Cholera spirillum.
Bacillus of diphtheria.	*Bacillus coli.*
Bacillus of leprosy.	*Bacillus dysenteriæ.*
Bacillus of malignant œdema (Koch)=Vibrion septique of Pasteur. Variable in reaction ; often Gram-negative in old cultures ; frequently needs prolonged staining.	Bacillus of Ducrey (=chancroid bacillus.
Bacillus of tetanus.	*Bacillus mallei* (glanders).
Bacillus of tuberculosis — prolonged staining required.	*Bacillus mucosus capsulatus.*
Bacillus Welchii (=*B. aerogenes encapsulatus*=*B. perfringens* =*B. phlegmonis emphysematosæ*).	*Bacillus pestis* (bubonic plague).
Many other putrefactive bacteria.	*Bacillus proteus.*
Micrococcus tetragenus.	*Bacillus pyocyaneus.*
Pneumococcus of Fraenkel.	Bacillus of Pfeiffer (influenza bacillus).
Staphylococcus pyogenes albus.	*Bacillus typhosus* (=Eberth's bacillus).
Staphylococcus pyogenes aureus.	*Gonococcus.*
Staphylococcus citreus.	*Meningococcus.*
Streptococcus pyogenes.	*Micrococcus catarrhalis.*
	Micrococcus melitensis.
	Pneumobacillus of Friedlander.
	Spirochætes of syphilis and relapsing fever.

CHAPTER XVII

METHODS FOR SPIROCHÆTES

A. General Methods: 1. Fresh Preparations—2. Films and Smears.
B. Methods for the Detection of Spirochæta pallida: 1. Dark-ground Illumination—2. Stained Films—3. Sections.

A. GENERAL METHODS OF EXAMINATION

1. Fresh Preparations

Blood spirochætes, particularly *Spironema recurrentis* (*Spironema duttoni*) of relapsing fever, may be seen by high-power examination with direct illumination. The active movements of this form, and the consequent agitation of the adjacent red blood corpuscles, catch the eye. Owing to the tenuity of spirochætes, trained eyes and carefully restricted illumination (see p. 340) are necessary to see the parasites themselves. For diagnostic purposes by far the best method is that of the *dark-ground illumination*, which strikingly reveals the shape and movements of these organisms. Use the dark-ground illumination as described on p. 156, and examine thin fresh preparations on slides of the correct thickness. Suitable optical combinations for searching for spirochætes are:

A 4 mm. ($\frac{1}{6}$ inch) objective and a high-powered ocular (*e.g.* a 4 mm. apochromatic and an 8 or 12 compensating ocular).

A 2 mm. ($\frac{1}{12}$ inch) oil immersion, *provided with the proper stop*, and a low or medium-powered ocular.

The 3·4 mm. ($\frac{1}{7}$ inch) oil-immersion objective, now made by many optical firms, gives excellent definition with the dark ground. Owing to its low numerical aperture a stop is not necessary. High eyepieces can be successfully used with it.

2. Films and Smears

Leishman and Giemsa's stains, used according to the standard technique, stain most spirochætes.

For Cytological Observations Dobell's method is to be recommended. Make films and fix, *without allowing them to dry*, in vapour of 2 per cent. osmic acid for thirty to sixty seconds. Then place the slide in absolute alcohol for ten to thirty minutes. Stain in Giemsa or Leishman according to the type technique (pp. 205 and 206 respectively). Wash in water, if necessary differentiate in dilute alcohol (in which case rinse in distilled water after differentiation), and allow to dry. Examine with the oil-immersion objective without mounting.

Note.—Dobell found that his material (*Cristispira veneris*) suffered great distortion if the films were allowed to dry before fixation with osmic acid, or if they were fixed direct in absolute alcohol.

B. METHODS FOR THE DETECTION OF SPIROCHÆTA PALLIDA

It is due to the extreme tenuity of this organism, the refractive index of which is very close to that of the blood plasma, and to its resistance towards stains, that it escaped notice until 1905, when Schaudinn and Hoffmann succeeded in staining it with Giesma's stain.

Ordinary stains — *e.g.* Delafield's hæmatoxylin — do not stain it either in films or sections. Heidenhain's iron-hæmatoxylin stains the organisms badly.

1. Dark-Ground Illumination of the Living Spirochæte in Tissue Fluids

This is by far the best method for diagnostic purposes. The staining methods (described later) are only to be recommended when the dark-ground technique is not available.

The following preliminary conditions are important :

The apparatus must be set up and adjusted as described on p. 156.

A good optical combination is a 2 mm. ($\frac{1}{12}$ inch) achromatic objective and a No. 2 Huyghenian ocular. But some workers prefer a high dry (4 mm.) objective and a high-powered ocular.

The aperture of the objective must be stopped down (see p. 158).

Clean slides of the right thickness and thin square cover-glasses ($\frac{7}{8} \times \frac{7}{8}$ inch or smaller) must be prepared.

A capsule of 0·9 per cent. NaCl and a platinum loop (or capillary pipette) are also requisite.

The examination of a syphilitic chancre is conducted as follows :

1. Moisten a swab in alcohol and roughly rub the lesion until it bleeds.

2. Wait until all bleeding stops, swabbing the chancre from time to time. A clear fluid exudate will appear after a few minutes from the abraded surfaces. (*Note : blood-stained exudate is useless.*)

3. Take up a drop of 0·9 per cent. NaCl with a sterile platinum loop, and place it on the centre of a slide.

4. Take up a drop of exudate from the periphery of the lesion with the flamed platinum loop, and deposit this on the drop of saline on the slide.

5. Repeat twice operation 4 (*i.e.* one loopful of 0·9 per cent. NaCl and three loopfuls of exudate).

6. Add a cover-slip and gently press it down with the handle of the platinum loop.

7. Examine for spirochætes with the dark ground, bearing in mind the five following points :

(i) The examination must be systematic—*i.e.* field by field—and is preferably done with a mechanical stage.

(ii) The *depth* of the saline-exudate mixture must be examined by continuous focusing up and down with the fine adjustment. The uppermost layers tend to be the richest in spirochætes.

(iii) Attention should be paid to the fluid immediately adjacent to air-bubbles in the preparation. Sometimes spirochætes are more abundant here than elsewhere.

(iv) Experience alone will aid you to distinguish between *Spirochæta pallida* and other spirochætes.

(v) At least two slides should be carefully examined before pronouncing a given specimen negative.

NOTES.—(1) If the suspected lesion be healed, it may be necessary to incise it with a sharp knife.

(2) The technique described above is only applicable when patient and dark ground are adjacent. If the specimen of tissue-fluid has to be transported, the most satisfactory plan is to take up the exudate with a fine Pasteur pipette, seal the end, and transport it in a plugged test-tube or piece of wide glass tubing.

(3) The same combination of objective and eyepiece should always be used, since the distinction between the syphilitic and other spirochætes is largely one of size.

EXAMINATION OF OTHER LESIONS

Mucous Patches in the Mouth.—Examine scrapings and exudate from these.

Cutaneous Eruptions.—Scarification and examination of the exudate as for the chancre.

Lymph Glands.—Examination of the fluid obtained by aspiration with a large-bore needle.

2. Staining Spirochæta pallida in Films

GIEMSA'S STAIN.—A really good sample of this is essential. It is best bought ready made up. The spirochætes are collected as described above.

1. Fix dry smears of the fluid or other material suspected to contain spirochætes in absolute alcohol for ten to twenty minutes.

2. Dry with filter paper.

3. Add 10 drops of the undiluted (stock) Giemsa solution to 10 c.c. of pure distilled water in a measuring cylinder. The previous addition of 5 to 10 drops of a 1 per cent. solution of pure sodium carbonate to the 10 c.c. of distilled water intensifies staining and is to be recommended. Mix with the minimum of shaking. Pour the staining mixture over the slide and leave for ten to thirty minutes. The older the

preparation, the longer the time required to stain syphilitic spirochætes.

4. Rinse by holding the slide, smear downwards, under the tap for a few seconds.

5. Dry with filter paper. Examine with the oil-immersion objective without mounting.

6. In the case of old, air-dried films, staining may often be advantageously prolonged (time permitting) to several hours or even overnight.

Spirochætes, reddish violet. Red blood corpuscles, leucocytes, pus cells, etc., stained as in a blood film, but more deeply.

Notes.—(1) The distilled water must be pure and glass-distilled.

(2) All glassware must be scrupulously clean.

(3) If the film is overstained (a rare occurrence), differentiate for one to five minutes in a dish of distilled water. Then blot and examine as above.

(4) The diluted staining solution loses its strength after use; therefore never attempt to use it again.

Hage-Fontana Silver Method.—This method is very useful on account of its rapidity.

1. Make thin films of the suspected material and allow them to dry in the air without warming.

2. Fix for one minute in Ruge's fluid:

Concentrated (40 per cent.) formol	20 c.c.	=Ruge's fluid.
Acetic acid	1 c.c.	
Distilled water	100 c.c.	

3. Wash rapidly (for about ten seconds) in running water.

4. Flood the slide with the mordant:

Phenol cryst.	1 grm.	=Mordant.
Tannic acid	5 grm.	
Distilled water	100 c.c.	

Warm over a flame until the fluid steams. Put aside for twenty to thirty seconds.

5. Wash for thirty seconds in running water.

6. Stain in an 0·25 per cent. solution of ammoniacal silver nitrate by warming the flooded slide over a flame till steam rises, and putting aside for twenty to thirty seconds.

The solution does not keep indefinitely.

Silver nitrate	0·25 grm.
Distilled water . . .	100 c.c.

Immediately after flooding the slide with this solution, add one drop of ammonia to it. A brownish scum should appear on warming the slide as above.

7. Rinse in water.

8. Blot.

Examine direct with the oil-immersion objective.

Spirochætes, black on a yellow background.

3. Staining Spirochætes in Sections

Levaditi's Silver Impregnation Method.—A reliable type method. The spirochætes in post-mortem material—*i.e.* tissues which have remained in the body for twenty-four hours or so after death—are well impregnated.

1. Fix thin pieces of tissue in 10 per cent. formol for twenty-four hours or longer.

2. Harden in 90 per cent. alcohol for twenty-four hours.

3. Remove the alcohol with distilled water. Complete hydration of the pieces of tissue is attained soon after they sink.

4. Impregnate in a 1·5 to 3 per cent. solution of silver nitrate for three to six days at 37° C. (I have generally found a 2 per cent. solution satisfactory.)

5. Rinse the tissues in distilled water for one or two minutes. This is important, otherwise heavy deposits of silver will be formed in the more peripheral parts of the blocks of tissue.

6. Reduce for twenty-four to forty-eight hours at room temperature in the following solution :

Pyrogallic acid . . .	2 to 4 grms.
40 per cent. formalin . .	5 c.c.
Distilled water . . .	100 c.c.

Twelve to twenty-four hours' reduction, taking 4 grammes of pyrogallic acid, is (I find) more than sufficient.

7. Wash in distilled water for ten or fifteen minutes.

8. Dehydrate in ascending grades of alcohol, clear in xylol, and embed in paraffin.

9. Cut thin sections—preferably of 4 to 6 μ.

10. Dissolve the paraffin in xylol and mount in balsam.

Spirochætes are impregnated jet-black; owing to the deposition of silver they appear thicker than they really are. Connective tissue is usually brown; in sections of brain and spinal cord, nerve fibres also are impregnated dark brown and must not be confused, when segments of them are contained in a section, with spirochætes. Cells in general are a pale transparent yellow. The outer layers of the piece of tissue are blackened by over-impregnation, and are useless for the identification of spirochætes.

Notes.—(1) Stages 4 to 6 are best carried out in the dark or in brown glass bottles.

(2) Alcohol-fixed material should be soaked in 4 or 5 per cent. formol for twenty-four hours. It is then treated according to the above technique from stage 3 onwards.

(3) Material fixed in Regaud's fluid or in other combinations of potassium bichromate and formol can be stained by this method.

(4) The sections may be counter-stained with safranin, neutral red, or Borrel's methylene-blue (or thionin, or toluidin-blue). But little is gained by this procedure, as the silvering process largely inhibits sharp staining.

(5) There exist rapid modifications of the above method, but I have never had results as good as by the slow technique. This remark applies especially to material which has lain for some time in formol; in such cases the spirochætes either fail to be impregnated, or do so but faintly.

But the following has been recommended for frozen sections, and enables one to examine tissues in the course of a day.

Ambruzzi and Strempel's Rapid Method (a Modification of Jahnel's Technique).—Applicable to *frozen* sections.

Make the thinnest possible frozen sections of formol-fixed material and treat them as follows in Petri dishes:

1. Pure pyridine, one and a half hours.
2. Wash carefully in distilled water.
3. 96 per cent. alcohol, one hour.
4. Distilled water, one minute.
5. Uranyl[1] sulphate ($UO_2SO_4 + 3\frac{1}{2}H_2O$)—5 per cent. aqueous solution. Treat for two hours at room temperature.

[1] *Not* to be confounded with uranium sulphate.

6. Rinse rapidly in distilled water.

7. Place in 2 per cent. silver nitrate for two and a half to three hours at 37° C.

8. Transfer to 100 c.c. of an 0·25 per cent. solution of silver nitrate to which 20 c.c. of a 70 per cent. solution of a solution of gum arabic are added. Then—

9. Add 5 c.c. of a 5 per cent. solution of hydroquinone. Mix this with the gum-silver solution ; reduction is complete in about ten minutes.

10. Wash repeatedly in water.

11. Alcohol.

12. Xylol.

13. Balsam or dammar.

Spirochætes, black ; background, variable from yellow to red or greenish. Results said to be fairly constant.

CHAPTER XVIII

METHODS FOR PROTOZOA

1. Introduction—2. Illumination—Methods for : 3. Rhizopoda ; 4. Sporozoa ; 5. Flagellata ; 6. Ciliata.

1. INTRODUCTION

This manual being primarily histological, only the methods especially applicable to those protozoa which are encountered in tissues are dealt with.

But since the four great protozoal groups—Rhizopoda, Sporozoa, Mastigophora (=Flagellata), and Ciliophora (=Ciliates)—have each parasitic forms which may be encountered in Man and the other vertebrates, the "skeleton" of protozoological technique may be fairly said to be provided.

Protozoological technique is a very refined variant of histological technique in which the film or smear method plays an important part. The sectional method is largely subordinated to the study of films. In clinical work especially is it almost entirely restricted to autopsy material. Of great importance also is the direct microscopic observation of living protozoa, and such "fresh preparations" form the basis of clinical protozoology. In the latter sphere, it is the body fluids (blood, lymph, etc.), and the fæces, which are most frequently examined for evidence of systemic or intestinal infection.

Protozoological methods are among the most difficult forms of microscopical technique.

2. ILLUMINATION

Artificial light, suitably screened, is to be preferred to daylight for this as for all other critical, high-power observations. A point which must be stressed is that the light must

be suitably reduced for examination of fresh preparations of protozoa. Diagnostic features of these delicate organisms are quite lost in a blinding glare. The best way to reduce the light, as advised by Dobell and others, is by racking down the condenser, *not* by partially closing the substage iris diaphragm. Any one who has had occasion to examine fæces for protozoa realises the truth of this statement.

3. METHODS FOR RHIZOPODA

The only representatives of this group which the worker in pathological histology may encounter either in the tissues or in the intestinal contents are various species of amœbæ.

(*a*) Fresh Preparations.—Both active amœboid and encysted forms can be observed by dissociating fragments of infected tissue in 0·9 per cent. NaCl or in Ringer-Locke solution. In the case of fæces, these must be diluted so as to reduce the amount of solid vegetable débris, etc., and to increase the transparency.

To make a preparation from this material, deposit first with the platinum loop a drop of 0·9 per cent. NaCl on a slide; then take up some of the stool and rub it up in the drop of saline. Add a square cover-slip and avoid air-bubbles in doing so. Examine with reduced illumination. Such preparations must be thin. Movements of amœbæ may be resuscitated in the case of tissues or fæces which have stood for some time by the use of a warm stage.

(*b*) Iodine Preparations.—Prepare the specimen as for (*a*), using a fair-sized drop of Lugol's solution (formula, p. 117)[1] in place of the saline. The nuclei of cysts are more easily seen after fixation and partial staining by this method. It is particularly useful in clinical pathology where it is important to distinguish between the cysts of pathogenic amœbæ and those of harmless commensals of the intestine.

(*c*) Stained Films.—Watch-glasses or Petri dishes are requisite. The standard method is to make cover-slip smears. Bear in mind, in this connection, that the charged cover-slip

[1] Many workers prefer a stronger iodine solution made by saturating 5 per cent. KI with iodine.

is fixed *face downwards* by allowing it to drop on to the fixing fluid. If the fluid does not contain more than 50 per cent. of alcohol, the cover-slips will float on it. It is more convenient to float your cover-slips—manipulation is far easier—than to sink them during fixation, staining, differentiation, etc. In the higher grades of alcohol, and in xylol, they will sink. Therefore, *either* treat the cover-slips *film downwards* in watch-glasses, *or* sink them *face upwards* in Petri dishes, when using high-grade alcohol or xylol. This avoids scratching the film.

Fixation and Subsequent Treatment of the Film

1. *Preparation of the Film* (applicable to all methods).—Make thin smears with the platinum loop of the material to be examined. Slight dilution with 0·9 per cent. NaCl may be necessary in the case of consistent fæces, but care must be taken not to dilute the material too much or it will not remain attached to the cover-slip.

2. *Fixation.*—Drop the cover-slip, face downwards, on to the fixative. *Never allow the film to dry from the moment you smear it on the cover-slip to the moment you mount it.* Films which are allowed to dry produce distortion—especially in unencysted amœbæ. Have the fixative ready in a Petri dish so that you can drop the films on to it as soon as they are made. With a little practice, several films can be dealt with simultaneously in one Petri dish.

Fix for ten to thirty minutes in *Schaudinn's fluid* :

Mercuric chloride, concentrated aqueous solution .	2 parts.
Absolute alcohol	1 part.

3. Transfer to 50 per cent. alcohol for a few minutes.

4. Remove any free mercuric chloride by treatment with 70 per cent. alcohol to which a few drops of iodine-alcohol have been added (sufficient to tinge the alcohol a light brown. Leave for ten minutes or longer.

5. Pass through 50 per cent. (and 30 per cent.)[1] alcohol.

6. Place in distilled water.

The films are now ready to be stained by one of the follow-

[1] These brackets indicate that such steps may be omitted for rapid work—*i.e.* for diagnostic purposes.

ing methods, for which the general procedure as applied to paraffin sections is observed :

Delafield's Hæmatoxylin (formula, p. 106).—Useful for rapid work.

1. Stain for five to ten minutes in this.
2. Rinse in water.
3. Differentiate in dilute HCl (0·5 per cent.) for a few moments.
4. Control with the staining microscope by inverting the cover-slip (face upwards) on a slide. If necessary, differentiate further.
5. Blue the hæmatoxylin by floating on tap water (or Scott's tap-water substitute (formula, p. 126), which is quicker).
6. Dehydrate in ascending grades of alcohol, *e.g.* (30, 50) 70, 90 per cent., absolute alcohol. Two to three minutes in each grade suffices.
7. Clear in xylol (a few minutes).
8. Mount in dammar or balsam.

Nuclei of cysts, amœbæ, etc., blue.

Heidenhain's Iron-Hæmatoxylin. — The standard method of the protozoologist. Finer details of amœbæ and their cysts are well shown by it. A detailed description of the method, as applied to sections, is given on p. 136.

1. Mordant the films by floating them on the surface of a 4 per cent. iron-alum solution for six to twelve hours.
2. Rinse in distilled water for a few seconds.
3. Float on an 0·5 per cent. solution of hæmatoxylin (formula, p. 108) for six to twelve hours.
4. Rinse for a few seconds in distilled water.
5. Float on a 1 per cent. solution of the iron alum, and differentiate to the right degree. This part of the process must be controlled frequently with the staining microscope. The film is rinsed for a second or two in distilled water before being examined. A 4 mm. ($\frac{1}{6}$ inch) objective or, better still, a water-immersion objective, should be used. With practice, differentiation may be speeded up by using a stronger (3 per cent.) solution.
6. Arrest differentiation by washing in tap water for ten to thirty minutes according to the thickness of the film.

7. Dehydrate, clear, and mount as with the preceding method.

Nuclei, black ; cytoplasm, in shades of grey.

Dobell's Modification of Mann's Stain.—This method gives excellent pictures of amœbæ and cysts. It also has the advantage of being comparatively easy to manage.

1. Stain for four to twelve hours in Mann's methyl-blue-eosin (formula, p. 111).

2. Rinse in distilled water for a few seconds.

3. Differentiate, controlling with the microscope, in the following solution :

70 per cent. alcohol . . . . . 100 c.c.
Saturated aqueous solution of orange G . . A few drops.
(10 drops of this to every 100 c.c. of alcohol is a good proportion.)

4. Dehydrate, clear, and mount as described on p. 343.

Nuclei, blue ; cytoplasm, pink to blue.

(*d*) Notes.—(1) In cases of suspected amœbic dysentery, systematic examination must be made of several "iodine preparations." If the stool be not homogeneous—*i.e.* composed of solid and liquid masses—slides prepared from the different portions should be examined. A negative examination should be repeated—if possible, daily, until a definite diagnosis is arrived at.

(2) An eyepiece micrometer is especially useful in dealing with protozoal cysts, since the identification of these is often partly based on their relative dimensions. The micrometer should be calibrated for a given objective (preferably the 2 mm. ($\frac{1}{12}$ inch) oil-immersion objective), and the same tube-length always adhered to.

(3) The fresher the stools, the better. Amœbæ encyst soon after keeping the specimen, while the cysts themselves are apt to undergo degenerative changes after a few days.

(4) In the tropics the isotonic saline used for diluting fæces should be filtered (or sterilised), to ensure against protozoal infection of the fæces from this source. Furthermore, if a specimen of stool be kept for more than twenty-four hours, in very warm weather, free-living protozoa may be found in it which were not present in it—except in the smallest numbers, and in the encysted state—immediately after the stool was passed (Dobell and O'Connor).

(*e*) Sections.—Fix in Bouin, Zenker, mercuric chloride-acetic, or mercuric chloride-formol. Formol-fixed material

is also useful. (For formulæ and use of these fixatives, see the chapter on Fixation, pp. 27 to 43.)

Make paraffin sections of (preferably) not more than 6 μ in thickness.

Suitable stains are :

Heidenhain's iron-hæmatoxylin—used as for histological sections (see p. 136).

Dobell's modification of Mann's methyl-blue-eosin—described above.

Delafield's hæmatoxylin and eosin—used as for ordinary histological sections (see p. 126). This method is rapid and useful for diagnostic purposes. The lesions produced by amœbæ are well shown by it.

4. METHODS FOR SPOROZOA

Coccidia

Coccidia are very rare either in the tissues or dejecta of man, but they are comparatively common in the dog, cat, and rabbit. The small, soft, white nodules, often seen in the liver of laboratory (and wild) rabbits are due to *Eimeria stiedæ* (*Coccidium cuniculi*).

(*a*) Fresh Preparations.—Examine dissociated preparations of the infected tissue in normal saline. In the case of fæces, emulsify as for intestinal amœbæ (p. 341).

(*b*) Stained Films.—Fix and stain as for amœbæ (p. 342).

(*c*) Sections.—Fix in Bouin's fluid, mercuric chloride-acetic, or mercuric chloride-formol. Ordinary 4 or 5 per cent. formol can also be used. Make thin paraffin sections.

Stains which satisfactorily demonstrate both parasites and lesions are :

Heidenhain's iron-hæmatoxylin.

Dobell's modification of Mann's methyl-blue-eosin.

Delafield's hæmatoxylin and eosin (for diagnostic purposes).

For the use of these, see the paragraphs on the staining of amœbæ in sections (p. 344).

Note.—Heidenhain's iron-hæmatoxylin counter-stained with Minchin's lichtgrün-picric acid (p. 353) gives a clear differentiation of the

different stages in the life-cycle of these parasites. So does the Dobell-Mann method, which also demonstrates very well the lesions caused by these parasites (coccidiosis of rabbit's liver).

Hæmosporidia

The following account, though specially written with regard to malarial parasites, is largely applicable (in so far as pure technique is concerned) to the other types of Hæmosporidia.

(*a*) Fresh Preparations.—The practised eye can detect and diagnose the species of malarial parasite from the study of fresh specimens. In the tropics the movements of the pigment granules of the parasites can be seen without the warm stage. In temperate climates the blood must either be examined immediately the preparation has been made or a warm stage must be used. Since high-powered objectives are essential, the warm stage should be one of the variety fitted with a condenser. The light must be reduced by manipulation of the condenser rather than the diaphragm. *When examining body fluids or fæces for protozoa of any sort, this reduction of the light is an absolute necessity.*

(*b*) Thin Blood Films.—This is the standard laboratory method for all hæmosporidia. The stains of Leishman, Giemsa, and Wright demonstrate the parasites admirably. Stain in the standard way (see pp. 205, 206, and 208). The unmounted film is examined with the 2 mm. ($\frac{1}{12}$ inch) oil-immersion lens and a low-powered ocular. But if you are searching for " Crescents " (the sexual forms of the malignant tertian malarial parasite—*Plasmodium falciparum*), you can work more rapidly with a 4 mm. ($\frac{1}{6}$ inch) dry objective. In this case the film *must* be mounted, either in cedarwood oil or in balsam.

(*c*) Ross's Thick Film Method.—This is an invaluable adjunct to the standard thin film method when searching for malarial parasites, and should be employed when negative results are furnished by the latter. It is also useful for the blood spirochætes of relapsing fever.

Proceed as follows :

1. Take up a large drop of blood in the centre of a slide,

then spread it out with a needle so as to form a square or a circle of 1 to 1½ cm. diameter.

2. Allow the slide to dry at room temperature, or in a current of warm air, or in the incubator. The time required to dry a thick film depends, of course, on the temperature and on the amount of moisture in the air. Two to four hours at room temperature in a dry atmosphere, or one to two hours in a dry incubator at 37° C. will suffice. The slide should be horizontal—at any rate during the earlier stages of the drying process.

3. Dehæmoglobinise the film by placing it face downwards (flat or on the slant) in a dish of cold distilled water.

4. Fix in absolute alcohol or alcohol-ether for five to fifteen minutes.

5. Stain in *one* of the following ways :

(*a*) *In Giemsa's stain* diluted in the standard proportion (*i.e.* 1 drop of stain for every c.c. of distilled water) for ten to thirty minutes. Rinse rapidly in distilled water. Blot *around* the film and dry at room temperature or in a current of warm (but not hot) air.

(*b*) *By Manson-Koch's Borax-methylene-blue Method* (Formula, p. 117). Stain for half a minute. Rinse well in tap water. Blot *around* the film and dry carefully above a Bunsen burner.

6. Examine with the 2 mm. ($\frac{1}{12}$ inch) oil-immersion objective and a low-powered eyepiece.

In such a film the red blood corpuscles are destroyed ; it is their débris and the fibrin which form the bluish, unorganised background. *By the Giemsa method*, leucocytes, distorted but recognisable, are seen in far greater numbers than in a thin film owing to the thickness of the dehæmoglobinised film. Although the corpuscles are destroyed, the malarial parasites are recognisable.

With Manson's method the leucocytes and malarial parasites are stained in shades of blue.

Gametocytes (of benign tertian and quartan) and of malignant tertian (" Crescents ") are well shown by the thick film method. The trophozoites (" Rings ") are extremely difficult —if not impossible—to distinguish *inter se* in the cases of the benign tertian, quartan, and malignant tertian parasites by

this method, since such important diagnostic differences as the size of the red blood corpuscles and the presence or absence of Schüffner's dots are obliterated by the laking of the red blood corpuscles.

Notes.—(1) Such a film must be protected from dust (which it attracts by reason of its viscosity) during the drying process. Likewise from flies which may eat out patches in it.

(2) If left for long in a damp warm atmosphere, bacteria may appear in it. These, of course, must be discounted in such preparations, as they are due to contamination.

(*d*) Practical Notes on the Blood Examination in Malaria.—Sometimes two or three thin films and even a thick film may be most carefully examined and yet no parasites found. Such a case does not necessarily imply that the patient is not malarial, especially if there be positive clinical evidence (a history of malaria, an enlarged spleen, etc.). Perhaps even the case, in spite of the negative blood examination, has been put on quinine and has improved. And a periodic temperature, which is amenable to quinine treatment, can only be interpreted as malarial in origin.

In relation to the above, the following facts must be borne in mind :

(1) Parasites in the peripheral blood of patients who have been receiving, or who have just received, quinine are frequently very scanty or not detectable microscopically, except in the case of the gametocytes (" crescents ") of malignant tertian malaria, which frequently persist in the blood for weeks and months in spite of quinine therapy.

(2) On account of the fact that the field of an oil-immersion objective is so small, and that even several hundreds of such fields only represent a tiny volume of blood, a blood film cannot justifiably be said to be *microscopically* negative until it has been examined, by a competent observer, for half an hour. This, at any rate, was the opinion of the late Sir Patrick Manson.

(3) Parasites are often more scanty at the onset (rigor stage) of a malarial attack than later. A film may be negative at this period and positive later on.

(4) The presence in itself of pigmented leucocytes—best seen in a fresh preparation—is practically pathognomonic of an active malarial infection. This pigment is nearly invariably confined to the large

mononuclear leucocyte, and is derived from the pigment of phagocytosed parasites themselves. Chemically it is hæmozoin (or melanin), and its histo-chemical reactions have been already described (" Pigments " ; p. 178). Usually, however, pigmented leucocytes are found in intense infections in which the observation of the parasites themselves is easy.

(5) From the above, the need for a systematic search, field by field, with a mechanical stage is evident.

(6) When a differential leucocyte count shows a relative large mononuclear percentage of 15 per cent. or over, malarial infection is to be suspected. This increase is largely confined to the interpyrexial period, since the high mononuclear percentage is often masked by the transitory increase of other white blood corpuscles during the leucocytosis of the pyrexial period.

Briefly summarised, the value of the blood examination in malaria is largely proportionate to the care expended on it. When quinine is taken it is usually extremely difficult—and sometimes impossible—to detect parasites.

(*e*) Malarial Parasites and Other Hæmosporidia in Sections.—It is of great importance to obtain autopsy material (especially in the tropics) as soon after death as possible. The most suitable fixatives are those which best preserve the shape of red blood corpuscles, *i.e.* :

Mercuric chloride-formol (formula, p. 37).

Five per cent. formol in 0·9 per cent. NaCl.

Absolute alcohol.

Embed in paraffin and cut very thin (preferably not more than 4 μ) sections.

The following staining methods are applicable :

(i) Delafield's hæmatoxylin and eosin (used according to the type technique, p. 129).

(ii) Eosin-Borrel blue (used according to the type technique p. 133).

(iii) Giemsa's stain. To obtain good results with this :

1. Fix preferably in absolute alcohol.

2. Embed in paraffin and cut thin sections.

3. Stain in Giemsa's solution, diluted in the proportion of 1 drop of stain for every c.c. of pure distilled water, for twenty to twenty-four hours.

4. Rinse rapidly in water.

5. Differentiate rapidly in 0·5 per cent. acetic acid until the section is pink.
6. Rinse in distilled water. Blot.
7. Dehydrate very rapidly in absolute alcohol.
8. Xylol.
9. Dammar or balsam.

Nuclei, dark red; red blood corpuscles, pink; malarial parasites, bluish; their chromatin, red.

5. METHODS FOR FLAGELLATA

Trypanosomes

The general technique is as for malarial parasites for purposes of identification. For protozoological study, more delicate methods are necessary.

A. GENERAL METHODS OF EXAMINATION

(*a*) Fresh Preparations may be usefully examined. Living blood trypanosomes displace by their active wriggling movements the adjacent red blood corpuscles. The dark-ground illumination admirably demonstrates these forms. (For technique of dark-ground illumination, see p. 153.)

(*b*) Thin Films.—These are made in the standard manner. *Leishman*, *Giemsa*, or *Laveran's Borrel blue* technique are good for demonstrating trypanosomes. With these methods the nucleus, blepharoplast, and flagellum are stained red, the cytoplasm bluish.

(*c*) Thick Films.—Make these according to Ross's method (p. 346), and stain with Giemsa or Leishman. Distortion of the parasites is apt to occur.

Notes.—(1) Trypanosomes in human blood are usually scanty, and prolonged examination of blood films is necessary. In cases of sleeping sickness, puncture of the enlarged cervical lymph glands often clinches the diagnosis. The fluid of the gland is taken up with a *dry* hypodermic needle and syringe, spread on slides, dried, and then stained by Leishman's or Giemsa's method. Failing this, lumbar puncture, particularly in the later stages of the disease, may be tried. The cerebro-spinal fluid is centrifugalised for half an hour, the centrifuge tube is

gently inverted, and the fluid poured off, Then smears, made from the deposit, are stained as above.

(2) Repeated blood examinations should be made in suspected cases; the parasites often—but by no means generally—are more easily seen in the febrile phases of human trypanosomiasis.

(3) In suspected cases which clinically appear to be sleeping sickness, but in which microscopic examination fails to demonstrate the parasite, the following are suggestive of infection with *Trypanosoma gambiense* or *Trypanosoma rhodesiense :*

An increase in the lymphocytes and mononuclears of the blood.

An increase in the lymphocytes of the cerebro-spinal fluid up to 100 per cubic millimetre.[1]

A tendency for the red blood corpuscles in a fresh preparation to be grouped in clusters instead of forming rouleaux (far from pathognomonic).

A decrease in the number of red blood corpuscles.

(*e*) Sections.—Fix thin pieces of tissue in :

Mercuric chloride-acetic acid.[2]

Mercuric chloride in saturated aqueous solution.

Zenker's fluid (formula, p. 36).

Formol does not favour staining with Giemsa's or Leishman's solutions. Embed in paraffin and make thin sections.

The following staining methods are good :

Eosin-Borrel blue (see p. 133).

Nicolle's carbol-thionin (see p. 321).

Giemsa's stain (see p. 206).

Leishman's stain (see p. 205).

Heidenhain's iron-hæmatoxylin (p. 136). When using this method, differentiation must be carefully controlled. A water-immersion lens is here of great assistance.

B. SPECIAL METHODS

Minchin's Methods.—In work of this sort, whether with reference to trypanosomes or other types of protozoa, it must be remembered that the reactions of protozoa in general towards fixatives, stains, and other reagents are very different from those of mammalian tissues. Furthermore, the methods

[1] Normal human cerebro-spinal fluid never contains more than 5 cells per cubic millimetre.

[2] $HgCl_2$ saturated aqueous solution . . 95 c.c.
Glacial acetic acid . . . . . 5 c.c.

by which the protozoologist attacks the problem of the structure of these forms are far more refined and difficult to work than the average technique for normal or morbid histology. The following account is a summary of the methods introduced by the late Professor Minchin :

Cover-slip smears are to be preferred to those on slides. This is because a cover-slip smear can be rapidly and equally fixed by dropping it on to the surface of the fixative. It is important to fix the smear (*e.g.* of blood) *wet*. Two chief methods are available : (1) fixation in osmic acid vapour ; (2) fixation by immersion in a mercuric chloride-containing mixture.

(1) *Osmic Acid Vapour.*—To 20 c.c. of a 4 per cent. solution of osmic acid add 1 drop of glacial acetic acid. Fix the film for thirty to sixty seconds face downwards *above* this solution. A glass slide or a paraffin block with a circular glass ring (cemented, in the former case) form a satisfactory chamber. *The film or smear must be fixed wet*—i.e. *immediately after making it.* Then transfer—without drying—to a watch-glass of absolute alcohol for twenty minutes or longer.

(2) *Immersion Fixation.*—Fix in Schaudinn's fluid (formula, p. 342) for half an hour. Drop the cover-slip, charged with its *wet* smear, into a solid watch-glass containing the fixative.

The *standard mercuric chloride-acetic acid* fixative (p. 351) is also good. It is used in the same way as Schaudinn's fluid.

After fixation pass through 50 per cent. and upgraded alcohols to 90 per cent. alcohol, in which the slides can be kept until you wish to stain them. The alcohol series should be graduated in stages of 10 per cent. difference—*i.e.* 50, 60, 70, 80, and 90 per cent. Ten to fifteen minutes' sojourn in each will suffice. If the fixative contains mercuric chloride, a few drops of a concentrated solution of iodine-alcohol should be added to the 50 or 60 per cent. alcohol bath to remove any free mercuric chloride.

Stain with *Heidenhain's iron-hæmatoxylin* (bearing in mind the general observations on the use of this method on p. 136) or in *Giemsa's stain.* The following points must also be observed when staining trypanosomes with iron-hæmatoxylin :

(1) To make successful preparations of trypanosomes by the iron-hæmatoxylin technique, the smear, from the moment of its fixation to

the time when it is mounted in balsam, *must never be allowed to dry*. Precise staining cannot be attained unless this practice is observed.

(2) Prolonged (overnight) treatment with 3 to 4 per cent. iron alum is followed by prolonged (twenty-four hours) staining in 0·5 per cent. hæmatoxylin.

(3) Differentiation must be very carefully controlled with a 4 mm. ($\frac{1}{6}$ inch) objective, or, better still, a water-immersion lens.

(4) Dehydrate in grades of alcohol ascending from 10 per cent. A counter-stain, recommended by Minchin and simple in use, is lichtgrün-picric acid. *Dip* the cover-slip, after dehydration in absolute alcohol, for one or two seconds into a tube of the following mixture :

Lichtgrün	1 grm.
Picric acid.	0·5 grm.
Absolute alcohol . . .	100·0 c.c.

Rapidly rinse it in absolute alcohol. Clear in xylol.

The iron-hæmatoxylin method demonstrates well the blepharoplast and flagellum.

Giemsa's stain should be used as follows :

1. Fix in osmic acid vapour followed by absolute alcohol.
2. Stain in Giemsa diluted in the proportion of 1 drop of stain to every c.c. of pure distilled water. The film should be face downwards (*e.g.* in a large watch-glass). Stain for three to eighteen hours.
3. Rinse in tap water followed by distilled water.
4. *Either* dry off *or* pass rapidly through three changes of acetone into xylol.
5. In either case mount in Canada balsam.

Note.—Giemsa's stain does not give good results (according to Minchin) after fixation in mercuric chloride mixtures.

Additional Observations.—Trypanosome smears, which are allowed to dry either before or after fixation, show less shrinkage than with never dried variety. This indicates that not only the fixative but also the subsequent processes to which the organisms are subjected (dehydration, staining, and clearing) are partly responsible for the loss in bulk.

Shrinkage, however, in a properly fixed and stained " wet preparation " is slight and seems to affect the whole organism equally—*i.e.* there is no distortion. The staining is also better in " wet preparations."

Parasites of the Genus Leishmania

(*a*) Fresh Preparations.—Not very instructive, owing to the scarcity of leucocytes in which the parasites are found.

(*b*) Thin Films.—Stain according to the standard technique (Leishman, p. 205, or Giemsa, p. 206). Often prolonged search is necessary to demonstrate the parasites, which are almost entirely restricted to the large mononuclears and polymorphs. Since there is always a leucopenia, two or three films must be searched in order to examine a sufficient number of these cells. The edges of the film, and especially the ends, contain the most leucocytes.

(*c*) Other Methods of Identification.—(i) In *Kala-Azar* (caused by *Leishmania donovani*), the parasite may be obtained by *splenic or hepatic puncture* when ordinary methods fail to reveal it. The latter is the least dangerous. The needle must be absolutely dry ; if it contains water, the endothelial cells and the parasites within them will be greatly distorted. Smears are made on slides and then stained with Leishman or Giemsa's stains.

Splenic puncture yields a positive result in nearly all cases of kala-azar.

(ii) A *pronounced* leucopenia—*e.g.* one of 2000 to 3000 per c.mm.—is the rule. A differential leucocyte count shows (usually) the relative numbers of the white cells not far removed from the normal. These two points are of aid in establishing a differential diagnosis between kala-azar and malaria.

The infantile form of kala-azar of N. Africa.—The above remarks are applicable to this variety of the disease.

In *tropical sore* (=Delhi boil, etc.) the parasite (*Leishmania tropica*) can only be obtained from the actual lesion. This should be scraped or punctured, the material spread on slides, and stained with Leishman's or Giemsa's stains. Another method is to snip out a piece of tissue with fine curved scissors, and section it as for sections of trypanosome-containing material.

(*d*) Sections.—Fix and stain as for trypanosomes (p. 351).

Flagellates other than Trypanosomes

Under this heading may be grouped those flagellates which are encountered in the intestine and vagina. Clinically

the free or encysted forms are found in the fæces or vaginal secretion.

(*a*) Fresh Preparations.—When examining fæces for flagellates you proceed exactly as with amœbæ (see p. 341). The dark-ground examination of a suitably diluted stool is sometimes advantageous in that it facilitates diagnosis of the particular type of organism, since the number and distribution of the flagella can be easily made out.

(*b*) Stained Films.—Fix wet films in Schaudinn's fluid. Stain, preferably, with Heidenhain's iron-hæmatoxylin, and differentiate very carefully. The flagella are stained by this method. The technique is as for amœbæ (see p. 342).

(*c*) Sections.—As for Trypanosomes (p. 350).

6. METHODS FOR CILIATA

The only forms of this group likely to be encountered by the histologist (or the clinical pathologist) probably all belong to the genus *Balantidium*. *Balantidium coli*, sometimes found in the intestinal mucosa (and occasionally in the adjacent lymph glands), is the agent of balantidiosis. It is a normal inhabitant of the large intestine of the pig and various species of monkeys.

(*a*) Fresh Preparations.—The motile (non-encysted) forms are only found in the intestinal contents. The characteristic cysts, however, are passed from time to time in the fæces of infected human beings.

Make fresh preparations as when searching for entamœbæ (see p. 341).

(*b*) Stained Films.—Fix and stain as for entamœbæ (see p. 342). Bouin's fluid is also a good fixative for *B. coli* (Langeron).

(*c*) Sections.—Fix pieces of large intestine or abdominal lymph gland in Schaudinn's or Bouin's fluids. Embed in paraffin and stain as for sections of other intestinal protozoa (see p. 344).

CHAPTER XIX

PARASITIC WORMS

1. Trematodes: (*a*) Fresh Preparations—(*b*) Whole Preparations: (i) Fixation; (ii) Staining—(*c*) Sections—(*d*) Larval Forms—(*e*) Ova.
2. Cestodes: (*a*) Fresh Preparations—(*b*) Whole Preparations: (i) Fixation; (ii) Staining—(*c*) Sections—(*d*) Larval Forms—(*e*) Ova.
3. Nematodes: A. *Intestinal Nematodes:* (*a*) Fresh Preparations—(*b*) Whole Preparations: (i) Fixation; (ii) Staining—(*c*) Sections—(*d*) Larvæ—(*e*) Ova—(*f*) Concentration Methods—(*g*) Trichinæ—B. *Nematodes of the Blood and Lymph Streams:* Adult Forms—Larval Forms: (*a*) Fresh Preparations; (*b*) Thick Films; (*c*) Thin Films; (*d*) Notes.

As with the protozoa, only the methods applicable to parasitic forms are here considered.

The technique to be adopted is largely dominated by three considerations. *Firstly*, the contractility of these forms is so great that special precautions are necessary during fixation in order to avoid extreme contraction. *Secondly*, much of the morphology—especially when identifying the parasite—is based on the study of whole preparations, stained or unstained, rather than on sections. *Thirdly*, the examination of the ova of parasitic worms, in fæces or urine, is clinically very important, since the various species can be recognised by their ova. Such observations are usually made on fresh preparations.

A general remark may be made here. If you wish to observe the movements of the living parasites, place them in normal saline solution warmed to 37° C. (*e.g.* in the incubator). Cestodes, especially, become very sluggish at temperatures much below that of the body.

1. TREMATODES

The parasites should be freed from tissue or alimentary débris by washing in normal saline.

(*a*) Fresh Preparations.—Examine the adult parasite between a cover-slip and slide (if small), or between two slides (if large). Or examine in a shallow capsule of saline with the binocular dissecting microscope.

(*b*) Whole Preparations.—(i) *Fixation.*—Place the parasites in a test-tube filled four-fifths with normal saline. Shake vigorously for a few seconds. This causes the worms to straighten out. Then quickly decant the upper half or third of the saline and fill up with the fixative. The most suitable fixatives for general use are :

Mercuric chloride in saturated aqueous solution.

Mercuric chloride-acetic acid (formula, p. 351).

Fixation by either of these fluids is effected in one-half to one hour. Then remove any mercury deposit in 50 per cent. alcohol to which sufficient iodine in alcohol has been added to make the fluid a light mahogany brown. The method of causing extension by shaking, due to Looss, is especially useful for the smaller species. The extension is due to muscular fatigue.

With larger species proceed thus : Place the parasite between two glass plates (or microscopic slides). Gently tie a loop near each end. Then fix in mercuric chloride-acetic acid, heated to *circa* 50° C., by quickly lowering the flattened parasite into a beaker of the fluid. After fixation, remove the iodine as above.

To store the parasites until you are ready to stain or otherwise treat them, transfer to 70 per cent. alcohol.

(ii) *Staining.*—Transfer the worms to a capsule of *Mayer's Paracarmine* (formula, p. 109). Stain for twelve hours or even longer. Then destain in acid-alcohol (concentrated hydrochloric acid, 0·5 c.c. ; 70 per cent. alcohol, 100 c.c.). Control the process of differentiation from time to time with the staining microscope. It is impossible to state the time required, since it varies greatly according to the nature and thickness of the material, the fixative, etc. etc. But usually you have to differ-

entiate for several hours. Dehydrate in ascending grades of alcohol (50, 70, 90 per cent.), leaving the object for at least half an hour in each of these grades. Complete dehydration in absolute alcohol (two hours to overnight). Clear in xylol (one hour or longer). Mount in balsam, which, if the object be thick, must not be too fluid. Harden the mount well on the hot plate.

The object should be a transparent red, the different tissues in varying shades of red.

NOTE.—In the case of very thick objects which tend to curl beneath the cover-slip, a spring clip—carefully adjusted as to tension—should be used to keep the object flat until the balsam has set. A small lead weight may be used instead.

Mayer's Hæmalum.—Another useful stain for whole objects. It is more powerful than paracarmine. For its formula, see p. 108.

Take the material down to distilled water. Transfer to a capsule of stain and leave for one to twenty-four hours. The thicker the object, the longer the time required. Then differentiate in dilute hydrochloric acid (0·5 per cent. in distilled water). Blue the stain by immersion of the objects in ordinary tap water for half an hour or longer, or in Scott's tap-water substitute (formula, p. 126). Dehydrate, clear, and mount as for paracarmine (*vide supra*).

The object is stained in shades of blue. The various tissues are well differentiated by this stain.

Delafield's or Ehrlich's Hæmatoxylin, used as for sections, but without a counter-stain, are useful for small objects.

The above notes on the staining of Trematodes are applicable to whole preparations in general.

(*c*) SECTIONS.—Fix in any standard fixative of good penetrative power—*e.g.* Bouin, Zenker, mercuric chloride-acetic, or Carnoy. Stain by standard histological methods such as hæmatoxylin (of Delafield or of Ehrlich) and eosin (p. 126), Heidenhain's iron-hæmatoxylin (p. 136), or Mann's methyl-blue-eosin (p. 134).

(*d*) LARVAL FORMS (*Miracidia, Sporocysts, Rediæ, Circariæ*). —Fix in a watch-glassful of mercuric chloride in saturated

watery solution. Then stain as in section (*b*). Observation of the living larvæ reveals many details which are destroyed by fixation, and is to be recommended.

NOTE.—Owing to the small size of these organisms the times for fixation, dehydration, etc., may be greatly reduced. Mount beneath a cover-slip supported by a black paper square or some such device to avoid crushing.

(*e*) OVA.—(i) *In fæces.*—Emulsify a platinum loopful of the stool in a little saline on a slide. Add a cover-slip and examine with a low-power such as the 16 mm. objective ($=\frac{2}{3}$ inch).[1]

(ii) *In Urine.*—Allow the urine to stand for a few minutes in a conical urine-glass. Then take up a little of the deposit with a fine pipette. Examine between slide and cover-slip as above.

NOTE.—In cases of suspected infection with *Bilharzia* (*Schistosomum*) *hæmatobium*, if examination by the above method is negative, resort to the following procedure : Instruct the patient to pass urine. Then to pass a few drops by straining. Collect the latter and examine with the microscope. The ova are always present, if the urine be so examined, in cases of urinary schistosomiasis.

2. CESTODES

(*a*) FRESH PREPARATIONS may be examined in saline between slide and cover-slip if small. Larger Cestodes may be examined alive in a large capsule of saline beneath the dissecting microscope.

(*b*) WHOLE PREPARATIONS.—Fixation of the worm between two glass plates by the method described on p. 357 is the best method. Mercuric chloride-acetic acid, heated to *circa* 50° C., is an excellent fixative. Long Cestodes are best cut into segments before being fixed.

Stain as for Trematodes (p. 357).

(*c*) SECTIONS.—As for Trematodes (see p. 358).

(*d*) LARVAL FORMS.—Cysticerci are fixed by gentle pressure between two slides as for adult Cestodes. The scolex can usually be evaginated by placing the Cysticerci before fixation

[1] Many workers prefer to emulsify the stool with a little iodine solution (see p. 341)

in warm saline in the incubator. Staining in paracarmine or acid hæmalum is indicated. For the method of using these stains, see p. 357. The cover-slip, during the drying process subsequent to mounting, should be very lightly pressed down either by one of the compressors sold for that purpose, or by a small weight.

(*e*) Ova.—These are only found in the fæces, since the adult forms of all Cestodes inhabit the digestive tract. Search for ova in the stool exactly as in the case of trematode eggs (see p. 359).

3. NEMATODES

The technique for dealing with these parasites is very special. This is the result of (*a*) the impermeable cuticle of these worms, (*b*) the difficulty of fixing them in extension, and (*c*) the fact that section cutting (on account of the tough chitinous cuticle) usually needs special precautions. The technique for Filariæ is dealt with apart, since different methods are requisite on account of their site (blood and lymph streams).

A. INTESTINAL NEMATODES

(*a*) Fresh Preparations.—Examine between slide and cover-slip after washing the worms in normal saline.

(*b*) Whole Preparations.—With the exception of the Micro-filariæ, Nematodes cannot be satisfactorily stained in bulk, since the cuticle behaves as a barrier towards stains.

Looss, after experimentation with many different fixatives, devised the following excellent method :

1. Place the worms in a rather wide test-tube filled about one-third full of water (or isotonic saline). Shake vigorously in order to free the parasites (and particularly their mouth parts) from fæcal débris. Pour off the supernatant fluid so as to leave a column only 1 or 2 cm. in height.

2. Carefully heat some 70 per cent. alcohol to between 50° and 60° C. A thermometer is hardly necessary, since the suitable temperature is indicated by bubbles beginning to ascend from the bottom of the vessel. Do not boil the alcohol, or serious shrinkage will be caused. Should it catch fire,

cover with any handy object and the flame will promptly die out.

3. Quickly decant the worms from the test-tube to the beaker (or saucepan) of hot alcohol. They will immediately die in extension.

If you wish to store the parasites, keep them in 70 per cent. alcohol.

4. Clear the specimens by placing them in a wide-mouthed tube or capsule containing a 5 per cent. solution of glycerine in 70 per cent. alcohol. Transfer the vessel to a thermostat (*i.e.* the paraffin bath) at 50° to 55° C. The alcohol slowly evaporates, and the objects are usually cleared after eighteen to twenty-four hours.

5. Mount in pure glycerine or in glycerine jelly (formula, p. 119). It is better to ring the cover-slip (*e.g.* with Leitz's "Deckglaskitt"). I have in my possession nine-year-old specimens of intestinal Nematodes still perfectly preserved in glycerine jelly.

The transparency induced by the glycerine is very marked, and even fine features of the anatomy of Nematodes may be successfully made out.

Notes.—(1) You can use warm Carnoy's fluid in place of 70 per cent. alcohol. But the results are no better.

(2) Occasionally shrinkage will occur soon after the worms are placed in the 5 per cent. solution of glycerine in 70 per cent. alcohol. In such cases the glycerine content must be diminished.

(3) In the case of thick Nematodes more powerful clearing has to be attempted. Clear as above in the glycerine-alcohol mixture, transfer to 90 per cent., followed by absolute alcohol. Then clear and mount in creosote. *Lactophenol* (formula, p. 120), recommended by Langeron, is another good mounting medium for Nematodes. Specimens are transferred direct to it after fixation in alcohol. In contrast with the glycerine-alcohol clearing technique, museum and other formol-preserved specimens may be cleared and mounted in this medium without shrinkage.

(*c*) Sections.—Fix material in hot 70 per cent. alcohol or in warm Carnoy's fluid. The chitinous cuticle of Nematodes tends to splinter and to disintegrate the underlying tissues during section cutting. Consequently the following precautions must be observed :

1. After fixation, either cut the worm into segments, or incise the cuticle in several places, so as to assist penetration. Dehydrate in 90 per cent. and absolute alcohol. Clear in cedar oil—*not* in xylol, which only increases the brittleness of Nematodes. Embed in hard paraffin (melting-point, 55° or 60° C.). Langeron advises Altmann's mixture, in which the following substances are melted together :

Paraffin, m.p. 60° C. . .	850 parts.
Stearin	100 „
Bees' wax	50 „

2. If the material breaks during sectioning, paint the block with collodion as recommended by Looss. For the technique, see p. 83. The method is laborious, but essential for cutting the mouth parts, copulatory bursæ, and longitudinal sections of Nematodes.

3. Standard staining methods can be employed. Delafield's hæmatoxylin and a suitable counter-stain give good results.

(*d*) Larvæ.—The larvæ are collected in normal saline and fixed just as for adult forms. Clearing and mounting are also conducted on similar lines. Owing to the thinness of the cuticle they may also be stained. Hydrochloric acid carmine (formula, p. 109) has the great advantage of penetrating well. Stain as follows :

1. Bring the larvæ after alcohol fixation into a tube, to which the stain is added after pipetting off as much of the alcohol as is possible. Stain for three to twenty-four hours.

2. Pipette off the stain and differentiate the larvæ with 0·5 per cent. hydrochloric acid in 70 per cent. alcohol. Control the destaining process by examining a few of the larvæ from time to time with the staining microscope. When sufficiently differentiated :

3. Wash the larvæ in two or three changes of 70 per cent. alcohol.

4. Transfer to the glycerine-alcohol mixture and clear in this according to the standard technique (p. 361).

4. Dehydrate in 90 per cent. and absolute alcohol.

5. Mount in glycerine or in glycerine jelly.

5. Clear in xylol.

6. Mount in balsam.

Note.—The best detail is obtained by the glycerine method, since the balsam clears the transparent and delicate larval tissues too powerfully.

Fixation in Carnoy's fluid should be of service and facilitate staining.

Delafield's hæmatoxylin, preferably after fixation in 70 per cent. alcohol slightly acidulated with acetic acid, stains the larvæ fairly well. But it should be used dilute and the staining-time prolonged (twelve to twenty-four hours). Differentiate in an 0·5 per cent. aqueous solution of hydrochloric acid, blue by immersion in tap water, and mount in balsam. (Euparal should be of use here.) Glycerine jelly is *not* suitable for mounting, since the hæmatoxylin fades in this medium.

(*e*) Ova.—Nematodes may be identified by their ova in the fæces.

(i) *Fresh Preparations.*—These afford the readiest means for clinical diagnosis. Emulsify with the platinum loop a little of the fæces in saline or water on a slide. The amount of water you dilute the material with depends on its consistency : the more solid the stool the more must it be diluted. Then add a cover-glass and examine systematically (a mechanical stage is a great convenience) first with the 16 mm. ($\frac{2}{3}$ inch), and then with the 4 mm. ($\frac{1}{6}$ inch) objectives. The light should be reduced, preferably by lowering the condenser, since, as pointed out by Looss, the outlines of the eggs are thereby rendered sharper. This author also advises the use of large slides, 75 × 36 mm. (3 × 1$\frac{1}{2}$ inches), and cover-slips of 47 × 28 mm. (1$\frac{3}{4}$ × 1 inch).

Several such preparations—three according to Looss—must be carefully examined before regarding a given specimen of fæces as free from Nematode infection. See also (*f*), "Concentration Methods."

(ii) *Preservation of Ova.*—Fix in hot 70 per cent. alcohol, clear in the glycerine-alcohol mixture, and mount in glycerine jelly as described for adult worms. Staining methods are not very satisfactory, since more detail is shown in unstained ova.

I have stained in Delafield's hæmatoxylin, after causing the ova to adhere to a cover-slip by thinly smearing it with a little undiluted

glycerine and albumen solution (see p. 77). The eggs are then fixed in Carnoy's fluid (formula, p. 39), stained, and mounted in balsam. But the unstained specimens give better detail.

(*f*) Concentration Methods for Intestinal Worms in General.—When direct examination of the stools fails to reveal either worms or ova, sedimentation of these should be attempted. In the cases of slight infection, especially if the microscopic examination be rapidly made, there is a possibility of not finding the eggs. Most of the methods of concentration depend on the fact that intestinal worms and ova are comparatively heavy when suspended in a fluid, and hence tend to sink more than vegetable débris, which can be decanted off.

Diarrhœic Stools. — Place in a tall cylinder and stir thoroughly. Then allow to stand for several hours—preferfably overnight. Decant the supernatant fluid and take up a drop of the sediment for microscopic examination.

Solid Stools.—Place in a vessel of suitable size but not too narrow. Fill under the tap and stir. After three minutes, decant. Refill with water, stir, allow to settle, and decant. The processes should be repeated three to six times in all. The débris at the bottom of the vessel are then searched for worms or their eggs.

(*g*) Trichinæ.—The adult (intestinal) forms are examined by standard methods. To find the larval encysted forms in muscle, a fragment is excised and examined between two thin slides in a little 0·5 per cent. acetic acid. With light compression the embryos are easily seen.

B. NEMATODES OF THE LYMPH AND BLOOD STREAMS

The technique for studying the smaller (larval) forms in the blood and lymph stream is largely hæmatological.

Adult Forms

To deal with these it is only necessary to apply Looss's methods for the intestinal Nematodes (p. 360 *et seq.*). A curious point, and one of practical importance, is that adult Filariæ,

when examined alive, swell up and ultimately disintegrate in normal (0·9 per cent.) saline. Apparently the concentration of NaCl which is isotonic with the tissues of these worms is between 1 and 2 per cent. Such strengths should therefore be used—but for as short a time as possible.

To study the worms *in situ* (*e.g.* in blood or lymph vessels), excise the tissue containing them, and fix and embed in paraffin in the ordinary manner.

Standard histological stains are indicated.

Larval Forms (Microfilariæ)

(*a*) Fresh Preparations.—Make a fresh preparation of the patient's blood (see p. 201). If you intend to examine, or to keep the preparation, for any time, it should be ringed with vaseline. Examine with a 26 mm. (1 inch) or 16 mm. (⅔ inch) objective. Systematic examination of such a preparation easily reveals the presence of Microfilariæ, which, in a properly ringed specimen, will remain alive for a week or more (Manson).

(*b*) Thick Films.—Make one or more thick films by the method described on p. 346. Smear the blood so as to make a large square of about 15 mm. (½ inch). Such a film may be kept indefinitely.

To stain it, use one of the following methods :

(*A.*) 1. Dehæmoglobinise the film by placing it face downwards in a dish of distilled water for a few minutes.

2. Then harden by immersion in absolute alcohol for five to ten minutes.

3. Stain in *Giemsa* by the standard technique (p. 206).

4. Dry the film rather than blot it.

(*B.*) Dehæmoglobinise as above, then stain in Leishman by the standard technique (p. 205).

(*C.*) *Manson's Method.*—1. Stain and dehæmoglobinise the unfixed film for one hour in the following solution :

Saturated alcoholic solution of basic fuchsin	3 drops.
Distilled water	30 c.c.

2. Rinse in water.

3. Examine with the staining microscope, and, if overstained, differentiate in 0·5 per cent. acetic acid.

4. Rinse in water.
5. Dry the slide.

(*D.*) *Looss's Method.*—Fix *wet* cover-slip films by dropping them on to the surface of 70 per cent. alcohol heated to 50° to 60° C. When cool, stain with Giemsa, with or without previous dehæmoglobinisation.

Note.—Either examine the slide without mounting it with a 3·4 mm. ($\frac{1}{7}$ inch) oil immersion and the lowest eyepiece you possess, or examine with a low-powered objective after making a thin smear of cedar oil over the film. You can also mount the dry smear in balsam, but it will fade.

(*c*) Thin Films.—Make these according to the standard technique for blood (p. 203). Do not make too large a film, so as to avoid the edges and ends of it being coextensive with the edges and ends of the slide. This is because Microfilariæ tend to be found at the borders of the film. Stain, *without* dehæmoglobinisation, by Giemsa's or Leishman's methods.

Examine and mount as for thick films. The thin film method, on account of its simplicity and the good results furnished by it, is particularly recommended by Langeron.

(*d*) Notes.—(1) For rapid diagnosis, either examine the patient's blood in a fresh preparation, or make a thick film and stain it after dehæmoglobinisation. By these methods you diminish the area of blood to be examined.

(2) Bear in mind that the larvæ of *Filaria bancrofti* are most numerous in the peripheral blood during the evening, whereas they are scanty or absent from blood films made during the day. Ten p.m. is a good time to make the blood examination.

(3) With *Filaria loa* the periodicity is reversed. Therefore make blood films from 10 a.m. onwards.

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NOTE.—The works referred to here only represent certain of the more important sources of information. But most of them contain very full Bibliographies. Those which quote the literature are marked (B.). It must also be pointed out that the only way to get into touch with the methods of any aspect of Histological Technique is to go through the literature oneself. That is an individual effort, and no list of references —short of one totally disproportionate to the size of this manual— could cover the ground.

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